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CHANGE IN THE CONCENTRATION OF REDUCING SUGAR IN BOVINE FETAL FLUIDS DURING GESTATION[1]

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(Southwest Foundation for Research and Education, and Trinity University, San Antonio, Texas)

INTRODUCTION

The placenta, an organ found only in mammals, facilitates the exchange of food materials and waste products between the blood of the mother and that of the fetus. Rather extensive analyses of the blood on both sides of the placental barrier for some of the major metabolic constituents, i.e., carbohydrates, fats, proteins, and the oxygen and carbon dioxide tensions, have been recorded (Barcroft, '46), which point out this nutritional role quite clearly. Since mammalian fetal development is internal, the fetal membranes and the fluids which they elaborate (Burger, '39) seem to have as their primary function the provision of an enclosed liquid environment in which development of the fetus may take place while protected from unequalized pressure to which it otherwise might be subjected during prenatal life. Since considerable embryonic development takes place before the formation of a physiologically mature placenta, it would seem that the fetal fluids might also serve as a vehicle for the transportation of nutrient materials from the food-rich endometrium to the embryo and that some similar role might persist throughout gestation. It seemed worth while to make sugar analyses of these fluids at various fetal ages to determine whether there was any correlation between age and sugar concentration. No published reports have been found which reveal such data for bovine fetal fluids. Barklay, et al. ('49) presented data on the total reducing sugar of sheep fetal fluids ranging in age from 49 to 140 days, but the number of analyses is too small to correlate sugar concentration with age. Reports for human amniotic fluid by Makepeace and Fremont-Smith ('31) and Cantarow, et al. ('33) are likewise inadequate in this respect.

[1]Aided by a grant from Swift and Co.

[2]On leave from the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts.

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Materials and Methods

Pregnant bovine genital tracts were obtained from local slaughter houses. The amniotic and allantoic sacs were exposed by dissecting away the uterine wall and 5 ml. of fluid were taken from each with a syringe within 20 minutes after the cow was slaughtered. The blood sample was collected in a small beaker as the cow was hung and bled from the neck.

The age of the bovine fetus was estimated by measuring the length in centimeters from the forehead to the rump and correlating this measurement with the data of Winters, et al. ('42).[3] This was greatly

TABLE 1.
Correlation of Fetal Age With Length
(Data from Winters, et al. 1942)

Age of Fetus (in days)	Forehead-rump Length (in centimeters)
45	3.08
50	3.85
55	4.67
60	6.6
70	9.4
90	16.4
100	18.8
110	24.0
120	27.1
130	31.2
140	32.6
150	36.8
160	43.7
170	48.5
185	54.0
200	58.5
215	70.0
230	73.0
245	82.0
260	87.0

facilitated by plotting length against age and reading the age directly from a graph.

Each of the 5 ml. samples of fetal fluid or blood was transferred immediately to a 75 ml. Erlenmeyer flask containing a tungstic acid solution (35 ml. distilled water, 5.0 ml. 10% Na_2WO_4, and 5.0 ml. 2/3 N H_2SO_4). This precipitated the serum proteins (Folin and Wu, '19), which were removed by Seitz filtration.

[3]This information is reproduced in Table 1 so that it may be more readily available to other workers in the field.

Two milliliters of the protein-free filtrate were placed in a Folin-Wu sugar tube and 2.0 ml. alkaline copper tartrate solution[4] were added. This mixture was placed in a boiling water bath for 10 minutes. After cooling for approximately two minutes in tap water, 2.0 ml. of phosphomolybdic acid reagent[5] were added to develop a dark blue color (Folin and Wu, '20) and the tube was placed in the water bath for an additional 5 minutes (Fiorentino and Giannettasio, '40). (This additional heating, which is a modification of the original Folin and Wu method, prevents fading and allows readings to be made for a considerable time after color development.) After cooling to room temperature the volume was brought to the 25 ml. mark on the Folin-Wu tube. A portion was transferred to a 13 x 100 mm. quartz cell (Cuvette No. 14-306) and the color intensity read in a Coleman Junior Spectrophotometer (Model 6A). The concentration of sugar present was read from a standard curve previously prepared. In all cases the instrument had been standardized against an accompanying glass standard at 43% transmission using a wave length of 610 millimicrons.

The data were grouped by months, except for a few late pregnancy samples, in an attempt to observe a trend in the measurements. The mean for each of these groups was calculated[6] and then the .95 confidence interval for the population, according to the method outlined by Hoel ('47), pp. 144-45:

$$\bar{x} - \frac{(t.05)\ (s)}{\sqrt{n-1}} < m < \bar{x} + \frac{(t.05)\ (s)}{\sqrt{n-1}},$$

where n = sample size

$\bar{x}$ = sample mean

s = sample standard deviation

m = mean of population

$t_{.05}$ = Student's "t" score for a probability of .05. Student's

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[4]Alkaline copper tartrate reagent may be made by placing 40.0 grams anhydrous Na_2CO_3 in a large flask and adding 400 ml. distilled water and 7.5 grams tartaric acid. Dissolve with the aid of heat, then add 4.5 grams $CuSO_4$, mix and let cool. Transfer to liter volumetric flask, dilute to mark and filter.

[5]To prepare the phosphomolybdic and acid reagent place 70.0 grams acid molybdic anhydride and 10 grams Na_2WO_4 in a large flask and add 400 ml. 10% NaOH and 400 ml. distilled water. Boil vigorously for 20-40 minutes to remove ammonia in molybdic acid and then cool in ice water. Add 250 ml. concentrated H_3PO_4 (85%) and transfer to a liter volumetric flask and dilute to mark.

[6]The writers gratefully acknowledge the assistance of Mr. James G. Stewart for his help in making these computations.

"t" test was used to determine significance differences between certain means.

RESULTS

Fetal fluid and blood samples were collected from 75 cows (table 2). Each cow is represented by a fetal age in column one, which in effect

TABLE 2.
REDUCING SUGAR CONTENT OF BOVINE FETAL FLUIDS AND MATERNAL BLOOD

Fetal Age (in days)	Milligrams hexose per 100 ml. fluid Amniotic	Allantoic	Blood
40			100
49	104	145	
50	123	146	
54	95	150	
64			144
66			264
68	94	260	
70			94
74	80	86	
75	82	218	
76			140
78			218
82	72	244	
85			83
86	68	308	
88	69	360	
88	59	384	
90	76	488	
92	98	496	
100	109	223	
104	84	460	
105	59	268	
106			248
106	63	520	
110			72
110	100	232	
110	72		
112	100		
112			65
115			320
120	108	—	
130	82		
130	92		
135	87		
136	159	360	
140	126		
142	125	218	
147	157		
147	142		
150	201		
151			82
156	206		
156	300		
158	156		

TABLE 2. (Continued)

Fetal Age (in days)	Milligrams hexose per 100 ml. fluid Amniotic	Allantoic	Blood
160	107		
162	228	324	
162	440		
168	212	276	128
168	224	492	
172	197	485	
174	415	430	190
175	196		
178	420		
180	400		
181	188	440	79
183	342		
184	356		
190	212		
196	213		
196	212		
198	176		
203	278		
203	74	140	
208	186		
210	129		
210	266	435	146
216	218	425	
220	280		
224	81		
224	156		
230	73	276	
236	89		
244	93		
257	196		

indicates the length of her pregnancy. All analyses made on blood or fetal fluids from a given animal are tabulated in parallel columns so that the information is arranged in order of increasing age. The horizontal lines separate the data into groups for statistical treatment.

Analyses were made on 62 samples of amniotic fluid, 29 of allantoic fluid, and on blood from 16 pregnant cows. In many cases samples of the two fluids were obtained from the same fetus and in a few cases blood for analysis was obtained from the parent cow. The trend of the monthly means for hexose content of the amniotic fluid (table 3, and fig. 1) indicates a slight decrease during the second month of gestation, when the mean sugar level was 75 mgs. percent, followed by a marked increase which reaches a maximum of 261.6 mgs. percent during the sixth month. The latter portion of the curve falls off sharply, but its significance is questionable. Few fetuses in the late months of

TABLE 3
SUMMARY OF STATISTICAL DATA

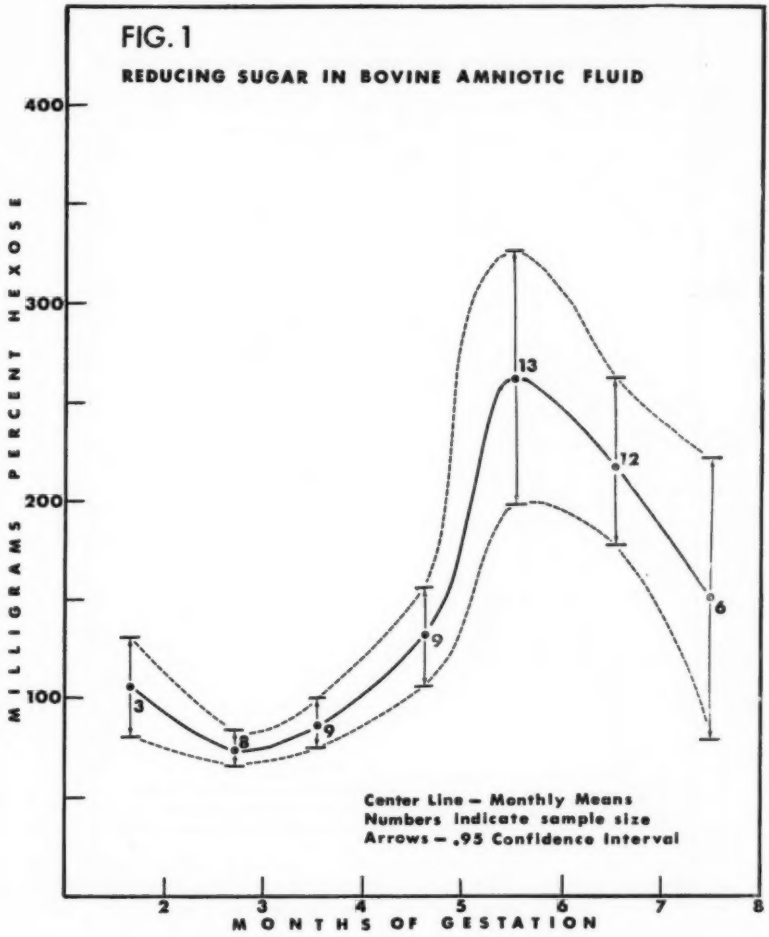
Month	Sample Time Avg. Months	n	$\bar{x}$	s	.95 Confidence Interval
		AMNIOTIC FLUID			
2	1.70	3	107.3	11.98	82.6<m<132.0
3	2.73	8	75.0	9.91	67.9<m< 82.1
4	3.55	9	88.1	18.21	76.1<m<100.1
5	4.65	9	130.1	37.15	105.7<m<154.5
6	5.56	13	261.6	120.74	199.5<m<323.7
7	6.55	12	219.3	77.95	177.0<m<261.5
8	7.50	6	149.5	77.43	79.7<m<219.3
		ALLANTOIC FLUID			
2	1.70	3	147.0	2.16	142.5<m<151.5
3	2.73	8	306.0	93.70	238.9<m<373.1
4	3.43	6	366.5	127.45	251.6<m<481.4
*	5.43	8	378.1	93.80	310.9<m<445.3
*	7.17	4	319.0	121.02	154.4<m<483.4
		BLOOD (PREGNANT COWS)			
		16	148.3	75.61	114.1<m<182.5
		BLOOD (NON-PREGNANT COWS)			
		6	100.8	28.92	74.7<m<126.9

*Special statistical group as designated in Table 2.

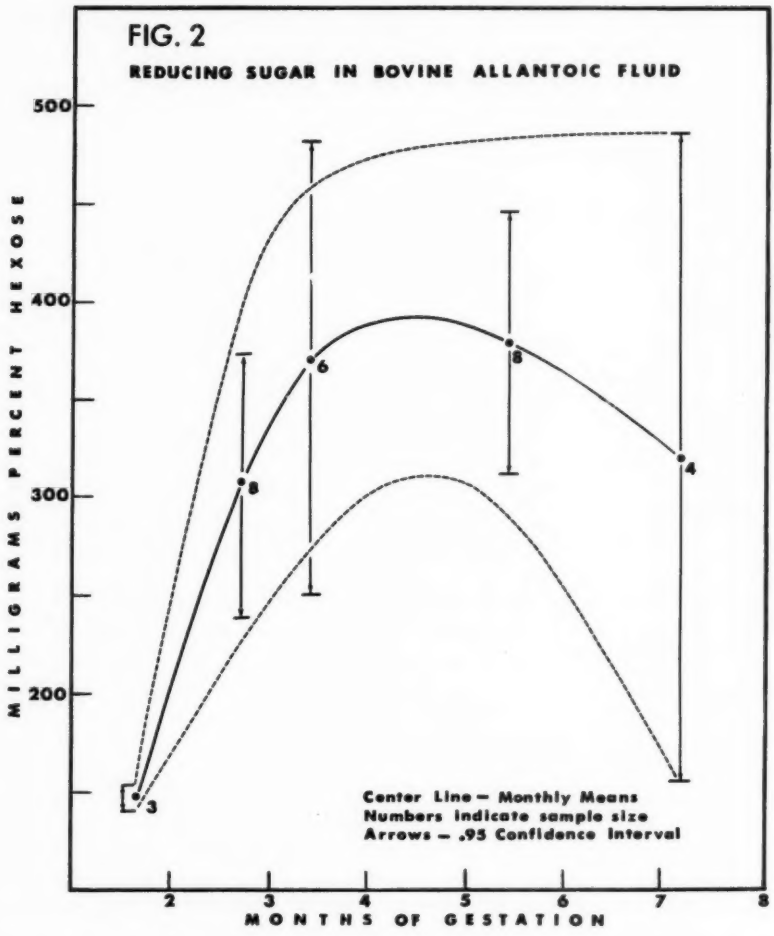
gestation were available, hence the curves do not extend to parturition. It is interesting to note that the mean sugar level of the amniotic fluid during the third and fourth months is significantly lower than the mean level for blood.

Since there were only 29 samples of allantoic fluid, data were not grouped strictly according to months, but somewhat arbitrarily (table 2). These groupings cover a longer period of time from the fifth month onward. A summary of the statistics on allantoic fluid is given (table 3), and the data are graphically represented (fig. 2). From a level of approximately 150 milligrams percent during the second month it rose to more than 350 during the fourth and sixth months of gestation. Since these data exhibit a very wide spread, the confidence intervals are only approximated by the curves. The trend appears to show a logarithmic rise reaching a significantly higher sugar concentration than that of the amniotic fluid, especially during the first half of pregnancy.

The sixteen gravid blood analyses presented a completely different statistical picture. No obvious trend in the amount of blood sugar was evident when blood sugar concentration was plotted against time.

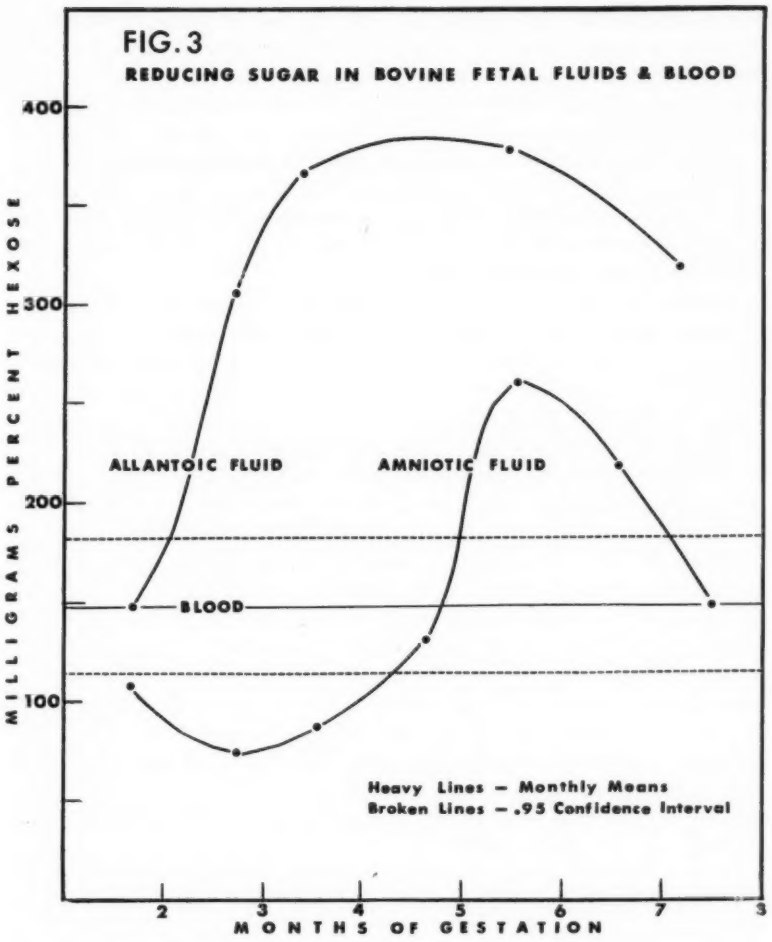


Analyses of blood samples from pregnant cows (table 2) were, therefore, treated statistically as a single group (table 3 and fig. 3). Six analyses of blood from non-pregnant cows were made in addition to the others, but the mean blood sugar was not significantly different from the mean of 148 mgs. percent for pregnant cows.



Discussion

The method of analysis used is not specific for glucose, although it and fructose are presumably the predominant reducing sugars present. Fructose has been associated with highly proliferative and motile cells, such as found in fetal tissues, tumors, cancer and sperm.



Needham ('50) refers to this as the "fructose problem". Itizyo ('34) found that of the total reducing sugar present in human amniotic fluid one tenth was present as fructose and the rest as glucose. More recently Cole and Hitchcock (cited by Barcroft, '46), in analyzing blood on both sides of the placental barrier in sheep, found that as much as

69 percent of the total reducing sugar present on the fetal side was fructose, whereas only 1 percent of the maternal blood sugar was fructose. Possibly some pentoses and disaccharides may also be present, but presumably in insignificant amounts, hence reducing sugar was calculated as total hexose.

Data on sugar analyses of human amniotic fluid by Makepeace and Fremont-Smith ('31) and Cantarow, et al. ('33) were too few to demonstrate a noticeable trend in the concentration during gestation. Accordingly, no valid comparison of our results can be made with those for human amniotic fluid.

Barklay, et al. ('49) made an analysis of sheep fetal fluids for reducing sugars and distinguished between the amount of fructose and non-fructose components. Their results for total reducing sugars approximate those found in the bovine in this investigation although it is difficult to evaluate their 14 analyses in this manner.

The great range in blood sugar observed may have been due to a peculiar physiological situation. The cows from which the blood samples were taken had fasted for 18 hours, but the digestive picture in the cow is so complicated that this fasting period is no doubt insignificant in view of the fact that some 72 hours are required for food to pass through the four divisions of the stomach (Dukes, '42). The dietary factor, therefore, could conceivably account for the large fluctuation noted in both pregnant and non-pregnant blood sugar levels. It may be noted (table 2) however, that low sugar levels were found scattered throughout pregnancy, which indicates that no conspicuously high titre is maintained during any prolonged portion of the period. From this evidence it appears that the relatively high concentration of reducing sugar present in the allantoic fluid during the third to seventh months and in the amniotic fluid during the sixth and seventh months is not derived directly from the blood.

It is evident (fig. 3) that the sugar rise in the allantoic or outer fluid precedes that of the amniotic or inner fluid, which may indicate a movement of sugar from the endometrium toward the fetus. The sugar may arise from a breakdown of glycogen stores of the endometrium, since glycogen granules have been shown to be released upon the administration of progesterone, which is quite abundant during pregnancy (Allen, et al. '39).

Another explanation is suggested by the findings of Huggett, et al. ('51). In a series of experiments involving the analysis of ewe's

maternal and fetal bloods for reducing sugars it was found that glucose was converted to fructose in the placenta. The quantity of glucose in the fetal blood was in nearly every instance lower than that of the maternal blood, indicating that a simple equilibrium existed. No measurable quantity of fructose was present in the maternal blood; however, the quantity of fructose always exceeded the amount of glucose in the fetal circulation. Injections of glucose into the ewe's blood resulted in a sharp rise in the fetal glucose and a slower but more lasting increase in the fructose. It is possible that the large quantities of reducing sugar found in the bovine fetal fluids could be attributed to the fructose present which seems to act as a reserve of hexose apparently out of equilibrium insofar as crossing the placental barrier is concerned.

Summary

Reducing sugar analyses were carried out on samples of bovine amniotic and allantoic fluids and on maternal blood from pregnant cows by a modified Folin-Wu method. In many cases both fetal fluids were collected from the same fetuses. Determinations were made on 62 samples of amniotic fluid from fetuses ranging in age from 40 to 257 days. Fetal ages were determined according to the method of Winters, et al. ('42). Average monthly reducing sugar levels ranged from 75 to 261 mgs. percent, the highest levels being attained during the sixth and seventh months. Twenty-nine samples of allantoic fluid from specimens 49 to 230 days old were analyzed in a similar manner. The mean level in the allantoic fluid from the third to the seventh month was approximately 350 milligrams percent; that for amniotic fluid significantly lower. The blood sugar mean for sixteen pregnant cows was around 150 milligrams percent, and did not differ significantly from the mean for non-pregnant cows. While there was a tendancy for trends to appear in the sugar concentration of the amniotic and allantoic fluids which could be correlated with fetal age, there seemed to be no correlation between the sugar levels in the fetal fluids and maternal blood.

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Growth, 1951, **15**, 225-239.

QUANTITATIVE GROWTH IN OBELIA COLONIES IN CULTURE

II. SEASON CHANGES IN RATES OF BASIC ACTIVITIES

E. MAINTENANCE; REGRESSION; CATABOLISM; INJURY

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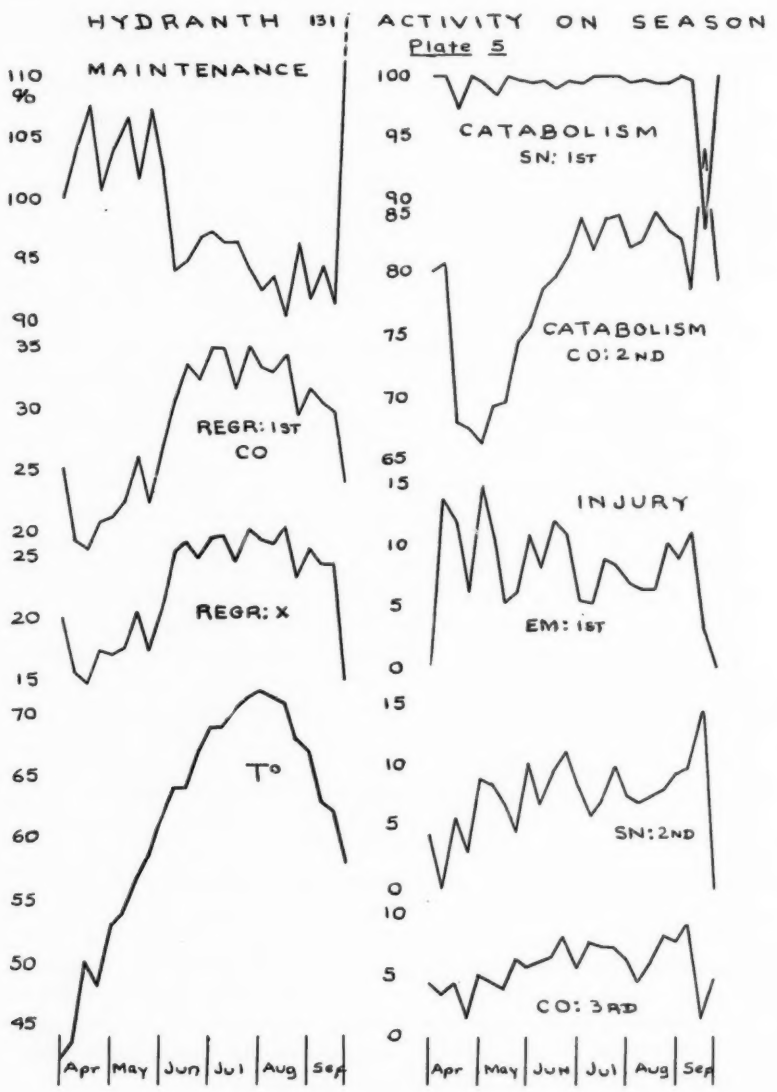
"Trust not too much in thine own notions."
ST. THOMAS À KEMPIS: IMITATIO CHRISTI

Rates, as usual, are of activity per 24 hours in 131 periods of 37 months of 7 years. The course of change in each activity over the weeks from April to September inclusive in all years combined is in Plate 1: together with that of temperature (T°). Each epitomizes the season change in the stated variable: since each is similar in overall to that exhibited in the years singly. Concern is with the overall course: not with non-general divagations therefrom. So such will usually be passed over. Correlations of period-to-period changes in rates with those in temperature are in Table 1. Variabilities are in Table 2: relative variabilities, and rates in overall in Table 2a. Procedures and rates are in the 1950 report as follows: Maintenance Intensity—Table 74: Maintenance Capacity—Tables 22, 24: Regression—Tables 76, 77: Catabolism—Table 79: Injury—Table 81. Temperature data are from Table 13 of the 1945 report.

MAINTENANCE

The feeding-excreting complete hydranth—composed of tentacles, gastro-vascular organ, and hydrotheca—is structural symbol of colony maintenance activity. Because colony energy exchange is mediated thereby, and because circa 71 per cent of the hydranths in colonies before culture are completes (1943: 1950). The activity is defined and measured by the numbers of completes in being in colony aggregates at stated times (1945). Two aspects will be analyzed: season change in intensity, and season change in capacity.

Intensity: The number of completes is index of maintenance in-


HYDRANTH ACTIVITY ON SEASON
Plate 5
MAINTENANCE
%
110
105
100
95
90
REGR: 1ST CO
35
30
25
20
REGR: X
25
20
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T°
70
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50
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CATABOLISM SN: 1ST
100
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CATABOLISM CO: 2ND
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EM: 1ST
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SN: 2ND
CO: 3RD
Apr
May
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Jul
Aug
Sep

tensity. But completes are continually lost by regression; and gained by organization (1943). So the best index in this material is the ratio of completes at end to those at beginning of the 24-hour time-slice. For increase is shown when the ratio exceeds 100: decrease when it is less than 100. And season change in these values is index of season change in intensity.

The Plate shows there is a time of intensity increase, and a time of intensity decrease. And that the activity tends to fall away as season advances. The gradation is evident in the years singly. Its somewhat obscuring in the data of all years combined is product of cancellations due to year-to-year differences in timing. Three factors of the course are conceivable: temperature, culture conditions, or colony ageing (maturing).

The course is not that of temperature. And period-to-period changes in intensity are not positively correlated with those of this factor. So temperature is eliminated.

Colonies are somewhat weakened by culture conditions: and expressions of activities are somewhat affected (1950: 1951a, b, c, d). The data include completes at end of culture: from colonies subjected to the maximal affect thereof. This enhances regression of completes to seniles: and may bring numbers at end to be less than at beginning (1950). Thus ratios under 100, or decrease in intensity, may be factored. But culture conditions exist in every period throughout the season: while decrease in intensity occurs only from 2nd June period on. And reaction to temperature, which is the usual result of colony weakening, is absent (1951, a, b, c, d). So culture conditions are eliminatable. Though it could be that while they are not responsible for the course, they may contribute to the extent of change therein.

The colonies increase in age and substance as season advances. Energy intensity is everywhere high in young organisms: and decreases as they increase in age and substance: because of ineluctably increasing entropy (Brody, 1945). Thus the course of season change in intensity is product of the endogenous factor of colony ageing: not to season or other change in exogenous factors. Corroborative is the fact ratios exceed 100 in the weeks of April—May and 1st June: are less from then on through June, July, August, and September.

The phase of higher ratios coincides with the time of most active increase in hydranth population. Colony maturing is based on increase in completes. More must be gained than lost at some time or other

for maturing to be accomplished. Early season is that time (1945: 1950). Thus ratios exceed 100 in the stated periods. Because of the endogenous factor of colony maturing.

The onset of lower ratios coincides with colony approach to maturity. As shown by the June levelling off of addition of new hydranths: and the antecedent increase in percentage of completes in colony populations (1945:1950:1951a).

The phase of lower ratios coincides with increasing maturity. Regression from functioning is a natural sequence of maturity. So colony maturing is a factor in the greater loss than gain in completes after 1st period June. Supporting which is the fact the ratios tend to decrease as season advances. Which is to say as the colonies increase in maturity. All is consistent. And again the Obelia record accords with that from other organisms: with all its implications in respect to the reliability of the data and their interpretations (1950).

Extent of change per period averages no more than 5 per cent. Thus the expression holds relatively steady within the period. This is evidence and demonstration of the principle that maintenance is primary in organismic economy (1945:1946:1950). Which, plus the fact of endogenous control, plus the fact the values cling close to 100 is why temperature affect is unseen (1951c, d).

Capacity: This aspect is measured by the percentage of completes in hydranth population-as-a-whole. Season change is had in colonies before and after culture (Table 22, 24:1950 report). The course in overall is the same in both and like that given in Charts 1 to 7 of the 1945 report. So no plotting is here given. The general picture is one of rapid then slower increase over the weeks of record. Two factors are evident: colony maturing and temperature.

The course as such is product of colony maturing: for four reasons. The colony is colony of hydranths, and the greater the percentage of completes the greater the maturity (1945: 1950). Capacity everywhere increases as viable substance increases: and colonies increase in such as they mature (1950). The increase in completes (capacity) slows down as maturity is approached. And capacity exhibits increase—not decrease—as temperature falls away after the August peak. All of which shows the course is endogenously not temperature determined.

But capacity increases as temperature rises: and period-to-period changes therein are positively correlated with those of temperature more often than not. Rise and fall in temperature generally factors

rise and fall in energy exchange (Brody, 1945). So the positive correlation is what should be. But temperature affects timing, duration, and extent of events: not their sequence. And here we are dealing with colony maturing which is sequence. And endogenous direction of sequence is everywhere impervious to change by exogenous factors in accustomed range (1945: 1950). So while extent of change in capacity may be temperature factored the course on season is otherwise determined. Other evidence of endogenous direction is the fact the course is not significantly altered by culture conditions. Despite there is culture-induced decrease in percentage of completes (1950), increase in variability (Table 2), and hint of increase in temperature affect (Table 1).

TABLE 1
CORRELATIONS BETWEEN ACTIVITY EXPRESSION AND TEMPERATURE

MAINTENANCE: BY MONTH

	Intensity						*Capacity*											
							Initial						*Terminal*					
	Periods			Years			Periods			Years			Periods			Years		
	S	O	N	S	O	N	S	O	N	S	O	N	S	O	N	S	O	N
AP	4	0	0	2	0	0	1	2	1	0	1	1	3	1	0	2	0	0
MY	7	6	7	2	1	4	8	8	4	3	3	1	12	4	4	6	1	0
JN	10	11	7	1	2	4	17	5	6	6	1	0	14	7	7	5	1	1
JY	9	11	8	1	2	4	11	5	12	4	2	1	11	7	10	4	1	2
AG	6	15	7	2	4	1	9	5	14	4	1	2	5	10	13	2	4	1
SP	7	7	2	3	3	1	6	4	6	4	2	1	6	5	5	2	2	3
TOT	*43*	*50*	*31*	*11*	*12*	*14*	*52*	*29*	*43*	*21*	*10*	*6*	*51*	*34*	*39*	*21*	*9*	*7*

BY YEAR

	Periods			Months			Periods			Months			Periods			Months		
'33	5	7	6	1	2	2	11	3	4	4	0	1	9	5	4	3	1	1
'34	5	8	2	1	2	2	9	2	4	4	1	0	6	4	5	2	1	2
'35	3	6	8	1	2	2	8	1	8	4	1	0	6	2	9	4	1	0
'36	7	4	6	2	0	3	6	3	8	3	0	2	11	3	3	4	0	1
'37	2	11	4	0	4	1	7	5	5	3	1	1	3	10	4	1	4	0
'38	11	5	3	3	0	3	6	6	7	3	3	0	8	4	7	4	1	1
'39	10	9	2	3	2	1	5	9	7	1	3	2	8	6	7	3	1	2
TOT	*43*	*50*	*31*	*11*	*12*	*14*	*52*	*29*	*43*	*21*	*10*	*6*	*51*	*34*	*39*	*21*	*9*	*7*

	REGRESSION						BY MONTH						CATABOLISM					
	CO: 1st						*SN: 1st*						*CO: 2nd*					
	Periods			Years			Periods			Years			Periods			Years		
AP	0	3	1	0	2	0	0	1	3	0	1	1	1	2	1	0	1	1
MY	5	10	5	1	5	1	1	0	19	1	0	6	10	6	4	4	2	1
JN	11	10	7	3	2	2	1	6	21	0	2	5	16	6	6	5	1	1
JY	11	8	9	3	3	1	1	2	25	1	1	5	10	9	9	3	2	2
AG	10	6	12	5	2	0	2	4	22	0	1	6	7	10	11	3	4	0
SP	9	3	4	4	1	2	1	2	13	1	2	4	8	5	3	3	1	3
TOT	*46*	*40*	*38*	*16*	*15*	*6*	*6*	*15*	*103*	*3*	*7*	*27*	*52*	*38*	*34*	*18*	*11*	*8*

TABLE 1 (Cont.)
CORRELATION BETWEEN ACTIVITY EXPRESSION AND TEMPERATURE

BY YEAR

	Periods			Months			Periods			Months			Periods			Months		
'33	8	7	3	2	1	2	0	1	17	0	1	4	8	4	6	3	1	1
'34	5	4	6	3	2	0	0	0	15	0	0	5	9	3	3	4	0	1
'35	3	6	8	1	3	1	0	2	15	0	1	4	9	1	7	4	0	1
'36	5	6	6	2	2	1	0	0	17	0	0	5	6	5	6	3	2	0
'37	8	1	8	4	1	0	2	4	11	2	2	1	5	6	6	1	2	2
'38	6	8	5	1	4	1	2	4	13	0	2	4	5	10	4	1	4	1
'39	11	8	2	3	2	1	2	4	15	1	1	4	10	9	2	2	2	2
TOT	*46*	*40*	*38*	*16*	*15*	*6*	*6*	*15*	*103*	*3*	*7*	*27*	*52*	*38*	*34*	*18*	*11*	*8*

INJURY: BY MONTH

	EM: 1st						*SN: 2nd*						*CO: 3rd*					
	Periods			Years			Periods			Years			Periods			Years		
	S	O	N	S	O	N	S	O	N	S	O	N	S	O	N	S	O	N
AP	2	2	0	1	1	0	1	1	2	1	1	0	2	2	0	1	1	0
MY	7	7	6	2	2	3	7	6	7	2	2	3	10	4	6	5	1	1
JN	13	10	5	4	2	1	16	6	6	6	1	0	11	9	8	4	3	0
JY	13	8	7	4	3	0	16	6	6	5	1	1	14	6	8	5	1	1
AG	7	11	10	2	3	2	11	10	7	2	3	2	6	13	9	1	3	3
SP	7	7	2	2	2	3	5	6	5	2	2	3	6	7	3	1	3	3
TOT	*49*	*45*	*30*	*15*	*13*	*9*	*56*	*35*	*33*	*18*	*10*	*9*	*49*	*41*	*34*	*17*	*12*	*8*

BY YEAR

	Periods			Months			Periods			Months			Periods			Months		
'33	6	7	5	2	2	1	4	6	8	2	3	0	6	7	5	1	2	2
'34	9	2	4	4	0	1	9	2	4	3	0	2	9	3	3	3	1	1
'35	3	8	6	1	3	1	6	6	5	2	1	2	4	6	7	2	2	1
'36	5	7	5	1	3	1	8	3	6	3	2	0	8	4	5	3	2	0
'37	8	4	5	2	0	3	7	6	4	2	2	1	6	5	6	2	1	2
'38	10	6	3	4	1	1	12	4	3	4	0	2	9	6	4	4	1	1
'39	8	11	2	1	4	1	10	8	3	2	2	2	7	10	4	2	3	1
TOT	*49*	*45*	*30*	*15*	*13*	*9*	*56*	*35*	*33*	*18*	*10*	*9*	*49*	*41*	*34*	*17*	*12*	*8*

Variabilities: Table 2a shows variability in intensity and capacity alike tends to be low in early season, high in late. The difference is due to colony ageing: maturing. Thus—

High rate means high endogenous control and vice versa. High endogenous control means low variability and vice versa (1951a). Intensity increases in young colonies (early season): decreases in old (late season). So season difference in variability in intensity is product of the endogenous factor of colony ageing. Capacity (% completes) increases as season advances because of drive to maturity. This is stronger in young immature colonies (of early season) than in old, more mature ones (of late season). So endogenous direction of capacity

is stronger in young than in old colonies. And variability is accordingly less. Thus season difference in variability in capacity is product of the endogenous factor of colony maturing. All is consistent and Q. E. D. Noteworthy is the fact April is the month of least variability.

Outside the fact 1934 is year of least variability in all expressions no tangible consistency is had in the seriations of relative variability by year: either as between the three expressions of maintenance, or as between their variabilities and their extents of expression. Probably because of year-to-year differences in timing, duration, and extent of seasonal progress in colony maturing. One would like to attribute the high variability in capacity before culture in 1939, 1935, and 1938; and the low in 1936 and 1934; to the high and low variability in temperature. Since capacity change is positively correlated with that of temperature. But such concordance is much less in the case of capacity at end of culture. Though it could be the relation here is scrambled by cumulative effect of culture plus other endogenous factors. But data are lacking. So conclusion is precarious. All that may be done is to note the possibility.

Regression

The complete hydranth eventually wears out and becomes a nonfunctionable senile. This structurally definitive step is mark of Regression. Its rate is the percentage of completes which go on to seniles in 24 hours (1943: 1950). Only the IP completes (initially present) express the activity in culture. Completes which come into being during culture are too young. Rate of regression of IP completes in all years combined on season is in Plate 1 (CO:1st). That of these plus the others is also given to complete the record (CO:x). Rate is here cut by circa 5 per cent. But the course is essentially the same. And the rate-temperature correlations in period-to-period changes do not differ much from those in Table 1. So analysis is based on the IP data.

The Plate shows regression starts low in early season: rises sharply to stay high through mid-season: after which it falls away. The course is product of hydranth ageing and temperature.

The proportion of older completes increases as season advances. Because colonies increase in completes: because completes continue as functioning entities for many days: and because addition of new hydranths decreases (1943: 1950: 1951a). Thus the approach of

completes to wearing out increases as the colonies grow older. And hydranth ageing conditions regression rise from early season low to mid-season high.

The course is like that of temperature. And while positive correlation of period-to-period changes in rate with those in temperature is absent in April and May, such is suggested in June, and definitely present in July, August, and September. Thus season change in regression is associated with that in temperature. It may be assumed that wearing out is slower in low temperature, faster in high. And that the older the complete, the weaker it is and hence more susceptible to reaction to temperature.

So then the low rate in April and May is attributable to combination of preponderance of young completes and low temperature: the June rise, to increase in proportion of older completes plus rise in temperature: the mid-season high, to proponderance of older completes and high temperature: and the late season decline, to increased susceptibility to temperature change resulting from weakening due to old age and closer approach to regression. The facts make the theory, not the theory the facts (Pareto, 1935). Thus the course of regression on season is product of the inter-woven factors of hydranth ageing and temperature.

Colony maturing is not involved. Period-to-period changes in regression are not positively correlated with those in numbers of percentages of completes. But it is possible that culture conditions contribute to the reaction, since the low rate of regression indicates the activity may be expressed late in the culture period of a time when colonies are somewhat weakened thereby (1950: 1951a, b, c, d).

The gamut of variability by month, 1934 the year of least variability:May the month, 1936 the year of greatest (Table 2a). The seriations, and the relations of relative variability to relative rate are too scrambled for comment. Save that the confusion may stem from the fact two varying factors are here major (hydranth ageing: temperature). Since seriations and relations are interpretable when but one (endogenous control) is major (1951a, b, c, d).

Catabolism

Once the hydranth is senile its substance is autolyzed and passed back into the colony via the hydroplasm until nothing is left but the empty hydrotheca atop its pedicel. The progression from senile to

empty is mark of catabolism. Its rate is the percentage of seniles which complete the change per 24 hours (Hammett and Padis, 1935: Hammett and Chapman, 1938: Hammett, 1943: 1950).

The activity has two expressions: catabolism of IP seniles and catabolism of seniles derived from IP completes during culture. The season course of each in all years combined is in Plate 1. Two types are shown: no change (SN:1st), and increase followed by decrease (CO:2nd).

The no-change course stems from the fact catabolism of IP seniles is so rapid season change in factors of influence has no effect. Rate is essentially 100 per cent of potentiality: 99.6 per cent of all IP seniles are completely catabolized during the 24-hour time-slice: 100 per cent in 114 of the 131 periods: more than 95 per cent on average in the remaining 17. Constatory is the low variability (Table 2), and the virtual absence of rate-temperature correlation (Table 1). The hint of negative correlation is unimportant to the course. Since autolysis is endogenous in origin and nature, and since the activity is here impervious to other influences, it is evident this course is endogenously determined.

The course of rate increase followed by decrease is possible because rate is slower. Rate is slower because time is used in regression of IP completes to seniles. This cuts time available for catabolism. Hence the percentage which complete catabolism in the 24-hour time-slice is less: and rate is less (1950).

The course is like that of regression (CO:1st). Because of this: because numbers of empties are positively correlated with those of seniles (1946): and because low rate of catabolism could derive from low rate of regression through late in-culture appearance of seniles, and high rate could derive from high rate of regression through early in-culture of seniles (1950): it would seem as if season change in catabolism rate were product of that of regression. But such decision requires positive correlation between the period-to-period changes in the two variables. This is lacking. Negative is had in 55 of the 124 changes: positive in but 51 (Table 79 vs. 76: 1950 report). Rate change in catabolism is independent of that of regression. So its course of change seems otherwise determined.

The course is like that of temperature (T°). And period-to-period changes in rate tend to be positively correlated with those in temperature (Table 1). As should be, since catabolism generally rises and falls

with rise and fall in temperature. It seems as if the course is temperature directed. This is fortified by the variability data (Table 2:2a).

"In continuity it is impossible to distinguish phenomena at their merging points: so we look for them at their extremes" (Fort, 1941).

TABLE 2
VARIABILITY IN EXPRESSION OF NON-GROWTH ACTIVITIES
(AD/M) 100

	BY MONTH					
No.	(6)	(25)	(28)	(28)	(28)	(16)
Month	April	May	June	July	Aug.	Sept.
TEMP.	6.0	4.5	3.9	4.3	4.2	3.7
MAINT.						
Intensity	2.9	4.1	5.5	5.4	5.5	5.7
Capacity						
Initial	2.7	3.1	3.2	4.0	3.0	5.3
Terminal	5.1	5.3	5.3	5.5	6.3	5.2
REGR.						
CO: 1st	8.5	18.9	14.5	14.1	15.1	17.6
CO: X	9.0	17.9	14.5	13.4	15.5	15.8
CATAB.						
SN: 1st	0.97	0.67	0.53	0.19	0.49	1.56
CO: 2nd	7.8	10.7	8.7	5.3	4.9	5.5
INJURY						
EM: 1st	41.9	56.2	48.9	52.6	60.6	62.5
SN: 2nd	100.0	100.0	77.4	58.3	56.5	68.7
CO: 3rd	71.8	61.3	64.1	49.9	64.6	57.5

	BY YEAR (Season)						
No.	(19)	(16)	(18)	(18)	(18)	(20)	(22)
Year	1933	1934	1935	1936	1937	1938	1939
TEMP.	7.0	5.4	9.6	6.7	8.6	9.9	13.8
MAINT.							
Intensity	5.8	4.8	5.9	7.1	8.0	4.9	5.2
Capacity							
Initial	4.4	2.2	5.5	3.8	3.1	4.6	5.6
Terminal	3.7	3.6	5.7	6.5	6.0	4.9	6.3
REGR.							
CO: 1st	19.5	15.8	16.1	28.8	19.7	19.3	16.5
CO: X	19.3	15.6	15.9	27.3	19.9	17.6	15.4
CATAB.							
SN: 1st	0.92	0.0	0.15	0.0	1.07	0.75	0.63
CO: 2nd	3.7	3.3	7.6	7.6	5.8	11.2	7.8
INJURY							
EM: 1st	74.7	50.8	54.9	36.7	62.0	50.0	69.7
SN: 2nd	107.6	74.3	35.6	68.8	62.6	62.9	70.3
CO: 3rd	66.2	33.4	19.7	21.0	49.7	65.7	58.9

This applies to these seriations. For while the gamut of month-to month and year-to-year differences in variability and rate differs in different cases; and the differences are at times too small to be significant (1st order rates especially): the months and years of high and low values are essentially the same for all. Thus the extremes of all are useable.

TABLE 2a
ORDER OF VARIABILITY AND RATE OF NON-GROWTH ACTIVITIES

	By Month	*By Year (Season)*
	TEMPERATURE	
Var	AP>MY>JY>AG>JN>SP	'39>'38>'35>'37>'33>'36>'34
T°	AP<MY<JN<SP<JY<AG	'39<'38<'33<'36<'37<'35<'34
	MAINTENANCE	
Inten.		
Var	SP>AG=JN>JY>MY>AP	'37>'36>'35>'33>'39>'38>'34
Rate	SP=AG<JY<JN<AP<MY	'37<'35<'38<'34<'36<'39<'33
Capac: Init.		
Var	SP>JY>JN>MY>AG>AP	'39>'35>'38>'33>'36>'37>'34
Cap	AG>SP>JY>JN>AP>MY	'34>'38>'35='33>'39>'36>'37
Capac: Term.		
Var	AG>JY>JN=MY>SP>AP	'36>'39>'37>'35>'38>'33>'34
Cap	AG>SP>JY>MY>JN>AP	'33>'34>'39>'38>'36>'35>'37
	REGRESSION	
CO: 1st		
Var	MY>SP>AG>JN>JY>AP	'36>'37>'33>'38>'39>'35>'34
Rate	AG>JY>JN>SP>MY>AP	'37>'36>'34>'35>'39>'33>'38
Co: X		
Var	MY>SP>AG>JN>JY>AP	'36>'37>'33>'38>'35>'39>'34
Rate	JY>AG>SP>JN>MY>AP	'37>'36>'35>'34>'39>'33>'38
	CATABOLISM	
SN: 1st		
Var	SP>AP>MY>JN>AG>JY	'37>'33>'38>'39>'35>'36='34
Rate	AP<JN<MY<AG<SP<JY	'37='39<'38<'33<'35<'36='34
CO: 2nd		
Var	MY>JN>AP>SP>JY>AG	'38>'39>'35='36>'37>'33>'34
Rate	AP<MY<JN<SP<AG<JY	'39<'38<'37<'36<'35<'34<'33
	INJURY	
EM: 1st		
Var	SP>AG>MY>JY>JN>AP	'33>'39>'37>'35>'34>'38>'36
Rate	JY<AG<MY=AP<SP<NY	'37<'34<'33<'38<'36<'35<'39
SN: 2nd		
Var	MY>AP>JN>SP>AG>JY	'33>'34>'39>'36>'38>'37>'35
Rate	AP<MY<AG<JY<JN<SP	'34<'37<'33<'38<'36<'35<'39
CO: 3rd		
Var	AP>AG>JN>MY>SP>JY	'33>'38>'39>'37>'34>'36>'35
Rate	AP<MY<AG<JN<JY<SP	'33<'37<'34<'36<'38='35<'39

The general trend is for variability to be high when rate is low and vice versa (by month and by year). This inverse relation is usually attributed to difference in endogenous control. Lower rate usually means lesser endogenous control, hence higher variability: and vice versa (1951a, b, c, d). But such interpretation here is questionable. Rate difference is temperature factored. And rate variability is high when temperature variability is high: low when it is low (Table 2a). Thus the high in rate variability is presumably product of the high in temperature variability: and the low is product of the low. And not of endogenous direction. Which fortifies the opinion the course of catabolism rate change on season is here temperature not endogenously directed. Since the season difference in rate variability is the same in direction as that in temperature (high in early; low in late) just as season difference in rate is same as that in temperature (low in early; high in late). All is consistent in overall. Sensitivity to temperature is perhaps opened up by culture conditions. In-culture appearance of these seniles is delayed by antecedent regression and its innate slow rate. Presumably to time when colonies are weakened thereby (1950a, b, c, d).

Injury

Pedicels without hydranths are found in colonies as collected; appear in colonies during culture. Such result from wave action in nature; handling during culture. The percentage is low; of same order of magnitude in both environments (2.4 vs. 2.7). This shows breakage has same basis in each; is not particularly factored by culture conditions. Brokens in culture derive from completes, seniles, and empties only; not from buds, halves, or three-quarters. Which shows they stem from weakening of the hydranth-pedicular jointure incident to growth completion. So breakage is endogenously, not exogenously, conditioned. It is mark of injury. Its measure is the percentage of IP completes, seniles, and empties found as brokens at end of culture (1943: 1950). Rate of each expression in all years combined is in Plate 1 (EM—1st: SN—2nd: CO—3rd).

Occasionally an empty hydrotheca or two is found floating in the culture dish; cast-offs from pedicels in which regeneration is starting a new hydranth. Occasionally pedicels are found on which the empty hydrothecae is partially disassociated. It might be inferred the pedicels called brokens are not products of injury, but of natural casting off

of empty hydrotheca preparatory to hydranth recurrence (1943:1950). To me this is untenable for most cases. Breakage is too low; stays in fairly constant overall range; fails to follow season change in maintenance, regression, or catabolism. Which it should (follow) if brokens were simply a step in recurrence. Moreover but 42.8 per cent of IP brokens go on to produce new hydranths (Table 36, 1950 report: 1951a). And empty hydrothecae found in culture dishes are added to the empties record, while a corresponding number is subtracted from the brokens. So to me the pedicels called brokens are predominantly just that.

It should be obvious the processes which take place in the jointure on growth completion differ from those which later prepare the distal pedicel for new hydranth production. Observation indicates the first is an entropic ageing polymerization: the second a rejuvenating reconstitution (1943: 1950). Excess in the former; but hardly the latter, could conduce to breakage. Thus injury is said to take origin in an endogenous activity which is separate and distinct from all others.

The activity exhibits no consistent change on season. The data of all years combined show no progressive trend (Plate 1). And Table 81 of the 1950 report shows rate rises in three of the seven years; falls in three; and changes not at all in one. The hint of rise in 2nd and 3rd order courses is unimportant here because due to fact extent of increase is greater than that of decrease in the respective years. The whole shows the need for records in several successive years if true conclusion is to be gotten. Certain coincidences are intriguing.

All three expressions show rise in the same years (1934: 1936: 1937); fall in the same years (1935: 1938: 1939): no change in the same years (1933). Which shows the basis for injury is same for all. As predicated above. Consider the years of rate change.

Rates are generally lower in years of rate rise than in years of rate fall (in 25 of 27 comparisons: 3 expressions x 3 years rise x 3 years fall). Which suggests endogenous control is less in years of rising rate. Support is had in other coincidences.

Period-to-period changes in lower rates are more frequently positively correlated with those of temperature in 8 of 9 comparisons (3 expressions x 3 years), while correlation of higher rates is positive in 5; negative in 3; absent in 1 (Table 1). Which suggests lower rates are more temperature factored. Hence less endogenously controlled.

Rate of regeneration from IP brokens; recurrence from completes,

seniles, and empties is generally greater in years of lower breakage rate (in 28 of 36 comparisons; 4 expressions x 3 years lower rates x 3 years higher rates: Table 36, 1950 report). This inverse relation suggests lower breakage rate is factored by the greater drive to rejuvenating reconstitution of the pedicels for hydranth replacement. Perhaps because less time is allowed for entropic ageing of the hydranth-pedicular jointure. Which, if true, would mean endogenous direction of the latter is less.

Other coincidences are worth noting even though their significance is obscure.

All three expressions are positively correlated with period-to-period changes in temperature in June and July (Table 1).

The years of lower rates are generally years of higher temperature and vice versa (in 7 of 9 comparisons: 3 years lower x 3 years higher: Table 2).

Variability runs high in all three expressions. That of 2nd order is generally highest by month and by year (seasonal): that of 3rd order is generally lowest by year (Table 2).

Relative variability by month and by year differs in each expression. Save that variability in all three is highest in 1933. The year of no progressive change on season (Table 2a).

These variabilities plus the fact years of lower rates are years of rate rise on season while years of higher rates are years of rate fall, yield the impression there is inherent in colonies a protective mechanism to keep breakage (injury) within non-destructive limits.

Summary and Conclusion

The non-growth activities exhibit the following rate courses on season.

Maintenance intensity decreases because of colony ageing (increasing entropy). It is not overtly affected by temperature. Maintenance capacity increases because of colony maturing (increase in viable substance). Its extent is temperature factored.

Regression rises as season advances and then falls away. The course is product of colony ageing, plus season change in temperature.

Rate of catabolism is innately so rapid it shows no progressive change on season. It is impervious to season changes in environmental factors. Rate of catabolism of seniles which appear later in culture increases as season advances and then falls away. The course is largely temperature directed.

Injury shows no consistent change in rate on season. This suggests presence of an internal protective mechanism against unwanted damage from external forces.

This completes recital of changes on season of rates of basic activities in Obelia. More could have been written. But the salient points are noted: and some detailed exposition of how the evidence may be evaluated.

The outstanding finding is that the course of constructive activities (growth and maintenance) in colonies unweakened by culture conditions is endogenously determined and controlled: while that of destructive activities (regression and catabolism) is exogenously directed. which is to say that endogenous control is superior to exogenous influence save when organism is weakened by age or oxygen and/or food deficiency. The shift is due to and expression of the ineluctable progress of increasing entropy. Which is as much a process of living as of non-living systems.

The whole, combined with the fact all activities are expressed in essential simultaneity in the same time-slices in the same environments, shows that the differences between activities in course of rate change on season, are not due to exogenous or environmental factors, but to those which are endogenous or intrinsic in nature. Which leads to the conclusion that, contrary to general assumption, it is not season change in environment which determines the change in rate of organismic activities on season, but the normal organism-determined march of life-cycle progression.

Citations

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EFFECTS OF WATER SOURCE ON TOXICITY OF MERCURIAL POISONS

II. Reactions of Allium Root Tip Cells as Indicators of Penetration

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The organic mercurials phenyl mercuric hydroxide and basic phenyl mercuric nitrate (PMOH and PMN), which are generalized poisons of respiratory enzymes, affect the root tips of *Allium Cepa* L. grossly and cytologically in a manner similar to colchicine. (Macfarlane and Schmock 1948), but chromosome stickiness and fragmentation also appear (Macfarlane et al. 1951). All reactions are produced in much lower concentrations than with colchicine and toxicity is affected by the aqueous medium seemingly because of differences in ionic balance (Macfarlane et al. 1951). The gross reactions give a rough indication of the cytological reactions. They are definite enough in any one solvent (aqueous medium) to suggest a method of bioassay for the intracellular penetration of the mercurials and of other growth arresting substances. (Macfarlane, 1950b).

In this report the following points are taken up:

1. Gross reactions in short and long treatments used to estimate penetration.
2. Correlation of cytological reactions with gross reactions and penetration.
3. Analysis of reactions with respect to cellular and tissue physiology.

Methods and Material. Bulbs of the selected onion variety Brigham Yellow Globe were used. They were germinated over 150-300cc. jars of each water or aqueous medium and treated in solutions with the same medium. Grown in the dark at room temperature, the roots were measured daily for 2 to 5 days until equilibrium was reached. Roots were treated when 3 to 8 cm. long (mostly 5 cm. i.e. 4 to 5 days old) and growing at an average of 1 cm. a day (Macfarlane et al. 1951).

Cytological reactions were observed in squash preparations fixed in acetic-alcohol 1 : 3 at various time intervals after an hour's treatment and during continuous exposure; and stained with acetocarmine, aceto-lachmoid, propriono-carmine or Feulgen's reagent after acid hydrolysis at 60° C. for 15 minutes. Where a counter stain was used best results were obtained with a very dilute mixture of light green and bismark brown in 95% alcohol. This stain gave light green cytoplasm and darker nucleoli. The matrix of mitotic chromosomes was also stained a purplish green, particularly when stainability of the chromatin was reduced by the mercury treatment. Permanent mounts were made in diaphane or euparol after dehydration in alcohol vapor and absolute alcohol.

1. *Gross Reactions and Internal Concentration of Poison*

The gross reactions produced by the mercurials resemble those caused by colchicine. They occur initially in the 2-3 mm. region of growth by cell enlargement (designated as region 2 of the root tip) immediately behind the 1-2 mm. of meristem (designated as region 1). Killing is first manifested in the penultimate 5 mm., the region of cell enlargement and of early differentiation (designated as region 3). The reactions have been arranged into four categories as already described (Macfarlane et al. 1951), namely:

Grade I—no arrest, some slight initial bending and hypertrophe in regions 2-3; growth sometimes stimulated. Associated with cytological reaction Grade 1.

Grade II—reversible arrest. Terminal swelling (c-tumor) in region 2 in one or two days, followed by resumption of normal growth. Associated with cytological Grades 2 and 3.

Grade III—arrest, and adaptation by slow growth and differentiation of meristem. Enlarged c-tumors followed in a few days either by recovery of slow growth in clubbed tips (IIIr) or by stasis (IIIs). Associated with cytological reaction Grade 3.

Grade IV—tissue of region 3 becomes flaccid, water-logged and necrotic in a few hours or days. Associated initially with cytological reactions Grades 3 and 4.

Differences in sensitivity of individual roots caused gross reactions in two or more grades in threshold concentration. Slow growing roots

and those under 3 cm. long were less sensitive than longer ones. (Macfarlane et al. 1951).

These gross effects are final equilibrium reactions in the enlarging and differentiating tissues above the meristem and differ from the cytological effects which, for the most part, are temporary and reversible in the meristem. As suggested by Levan (1949), the concentrations of poison that cause Grade I and II reactions are called subnarcotic, those causing Grade III narcotic, and those giving Grade IV lethal.

Toxicity is a function of time as well as of concentration. It was shown that similar effects were produced by an hour's treatment and prolonged exposure to a weaker solution of PMOH or PMN (Macfarlane et al. 1951). When the concentrations which gave parallel reactions were compared, it was found that the fraction of the stronger solution that was required in long treatment varied with the grade of gross reaction. The curves obtained by plotting these fractional dilutions of PMOH in four media are shown in Figure 1. In each water

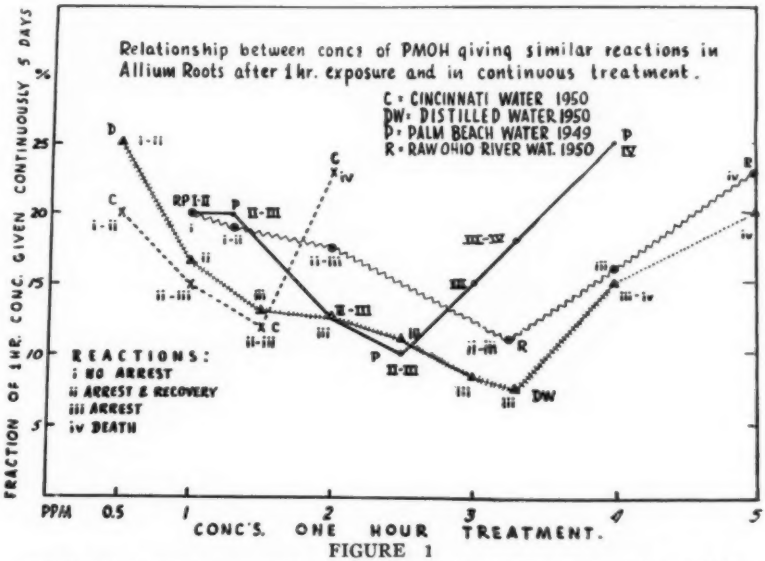
Relationship between concs of PMOH giving similar reactions in Allium Roots after 1 hr. exposure and in continuous treatment.
C = CINCINNATI WATER 1950
DW = DISTILLED WATER 1950
P = PALM BEACH WATER 1949
R = RAW OHIO RIVER WAT. 1950
REACTIONS:
i NO ARREST
ii ARREST & RECOVERY
iii ARREST
iv DEATH
FRACTION OF 1 HR. CONC. GIVEN CONTINUOUSLY 5 DAYS %
25
20
15
10
5
PPM 0.5 1 2 3 4 5
CONC'S. ONE HOUR TREATMENT.

FIGURE 1

Relationship between concentrations of PMOH giving similar gross reactions in Allium roots after 1 hour exposure and in continuous treatment.

the solutions which produced Grades I and IV in continuous exposure were 20-25% of the concentration which gave these reactions in one hour, but a greater dilution was necessary to obtain parallel reactions in Grades II and III. In Cincinnati tap water (Figure 1, C), Palm Beach tap water (PB) and raw Ohio River water (R), the lowest point on the curve corresponds to the highest concentrations of PMOH that permitted some recovery of normal growth, i.e., the upper threshold for Grade II, and was around 10% of the one hour concentration for this effect. In distilled water (Figure 1, DW) the proportional concentration fell rapidly and continued to fall in concentrations that gave only Grade III. In prolonged treatment all vital processes appeared to be throttled down in DW so that the roots merely subsisted in a range of concentrations; some grew slowly in Grade IIIr while some were static in Grade IIIs and as the concentration increased more roots became static. The curve therefore flattens out, falling slowly to 7.5% of 3.3 ppm. It then rises steeply to 15% of 4 ppm, the 50% lethal dose concentration where some roots survived in Grade IIIs and some died (Grade IV). In river water some recovery (Grade II) occurred after treatment in a range of concentrations from 1 ppm to 3.3 ppm (11×10^{-6}M) for an hour and from 0.25 ppm to 0.4 ppm (1.4×10^{-6}M) in continuous exposure, so the curve falls gradually to 12% at 3.3 ppm.

No direct proof was obtained that the internal concentration of poison in the root cells reached equilibrium with the external concentration in one to five days of exposure. However, if we admit only that a larger proportion of the poison penetrated the cells from stronger solutions, then these curves indicate a progressively reduced permeability to PMOH in an hour's exposure in concentrations which arrested growth, and also a rapid premortal rise in permeability. The curves therefore correspond with the reversible reduced permeability which has been reported for cells treated with narcotics, urethane and other respiratory poisons (Heilbrun 1943).

The fraction of the minimum lethal dose (MLD) which permitted recovery in Grade II is shown for each solvent and treatment in Table I. With the short treatment roots recovered after 62.5-75% of MLD in all solvents except distilled water (DW) where more than 20% MLD caused permanent arrest.

In continuous exposure roots adapted and recovered normal growth in concentrations from 25% to 33% of the MLD, except in DW

TABLE I
MAXIMUM CONCENTRATIONS OF PMOH IN PARTS PER MILLION (ppm) TOLERATED BY ALLIUM ROOTS FOR RECOVERY TO NORMAL (Grade II) GROWTH RATE IN RELATION TO THE MINIMUM LETHAL DOSE (MLD) IN FOUR SOLVENTS AND 2 EXPOSURES. 1949 DATA.

Exposure time		1 Hour			Continuous	
Aqueous solvent	MLD	Max. for Grade II	% of MLD	MLD	Max. for Grade II	% of MLD
*Cincinnati tap	2	1.5	75	0.5	0.15	30
Palm Beach tap	4	2.5	62.5	1.0	0.25	25
Ohio River	5	3.3	66	1.2	0.4	33
Distilled	5	1.0	20	1.0	0.15	15
*same, summer 1950	2.4	1.5	62.5			

where only 15% could be tolerated. The greater sensitivity in prolonged treatment may be due either to (1) an accumulation of the enzyme inhibiting effects of the poison, or (2) a dissipation or reversal of these effects upon return to plain water after the short exposure to higher concentrations. Both factors may have operated.

The fifty percent lethal dosages in 1949 were 80-90% of the MLD in all solvents for an hour's exposure, and 60% to 83% of the MLD in continuous treatment. There was thus less difference in tolerance between short and long exposure with respect to Grades III and IV reactions than for Grade II. (The 50% lethal dose in Cincinnati in 1950 was 75% of the MLD as against 90% in 1949.)

Discussion of Gross Results

The results show that the aqueous solvent affected the resistance of the different tissues of the root differently. In DW the synergistic effects of anoxia and hypotonicity may operate to decrease both adaptability in long exposure and penetration during the hour; while in river water the organic matter present may have antagonized some of the mercurial. (Oxidizable organic material in the Ohio River water was 5-6 ppm.) Cincinnati tap water contained slightly more calcium and chlorides than Palm Beach or River waters (Macfarlane et al. 1951). Calcium decreases permeability and this will tend to reduce turgor and lead to slower intake of water. The organic mercurials reduce oxygen uptake by living cells (Cook et al. 1946) and the resulting depression will also decrease turgor and retard growth by cell enlargement. Figure 1 shows that in Cincinnati water the higher sublethal concentrations of PMOH were particularly deleterious to tissues of regions 2 and 3; they also arrested the differentiation which was a

prominent feature in DW. In continuous exposure in Cincinnati water some Grade IIIr (with up to 50% normal growth rate) resulted in concentrations from 0.2 ppm to 0.33 ppm, but the static Grade IIIs was practically eliminated, hence the steep rise in the curve in the premortal period.

If as suggested, the curves correspond with a reversible reduced permeability in the presence of PMOH, then this response appears to have failed rapidly in tap water when the upper threshold concentration for normal recovery (Grade II) was reached. It ceased more gradually in river and in distilled water.

Correlation of Cytological Reactions With Gross Reactions and Concentrations of Poison

Grades of Cytological Reactions and Related Abnormalities.

Four grades of cytological reactions have been described briefly (Macfarlane et al. 1951). As mentioned above, they correspond fairly well with the gross reactions of the older tissues (see Table II) and have been designated by Arabic numerals to distinguish them from the grades of gross reactions. They were analysed usually from 1 to 24 hours after the beginning of the treatment in the period during which the cells above the meristem were often inactivated (not enlarging). Unlike the gross reactions, cytological reactions occurred as an orderly series of events from Grade 1 to Grade 3 which were usually reversible. Details of the chief abnormalities caused by these treatments are given for use in comparison with similar effects produced by specific enzymic poisons, x-rays and malignant changes.

Grade 1—slowing of all phases of nuclear and cell division with a tendency for arrest in telophase and prophase (Table VII).

Grade 2—inefficient achromatic spindle, impeded chromosome movements and lack of synchronization throughout mitosis (involving also the nucleoli and formation of nuclear membranes by telophase daughter nuclei); gives rise (a) to bi- and multi-nucleate cells through lagging chromosomes or suppression of cell-plate formation, and to (b) polyploid restitution nuclei (Macfarlane and Schmock, 1948) after failure of the external spindle. Aggregates of chromosomes in interphase lead to karyorhexis in fixed material. (Somatic segregation (distributive mitosis) and other irregularities reported by Huskins and co-workers (Huskins and Chen 1950) have been observed in cells in Grade 2

TABLE II

CYTOLOGICAL EFFECTS OF PMOH AND PMN ON ALLIUM ROOT CELLS AT VARIOUS INTERVALS AFTER AN HOUR AND DURING

TABLE II

CYTOLOGICAL EFFECTS OF PMOH AND PMN ON ALLIUM ROOT CELLS AT VARIOUS INTERVALS AFTER AN HOUR AND DURING CONTINUOUS TREATMENT IN CINCINNATI TAP (C) AND PALM BEACH TAP (PB) WATERS AS SOLVENTS AND MEDIA.

± Rare, 1-3 per tip; + Few, 2 in 2 to 3 hp fields; ++ Normal or 2-5 each hp field.

Poison in ppm	Hrs. after 1 hr. exposure	In Cincinnati City Tap Water						In Palm Beach City Tap Water						Hrs. after 1 hr. exposure	Poison in ppm
		2n Divs.	over 2 nucleoli	Polypl. Divns.	c-mitosis	Grade of Reactn. Cytol.	Gross	2n Divs.	over 2 nucleoli	Polypl. Divns.	c-mitosis	Grade of Reactn. Cytol.	Gross		
PMOH															PMOH
0.5	24	+	+	+	—	2 (recov.)	I-II								
1.3	24	—	—	—	—			+	±	±	±	3-2 (recov.)	II-III	24	1.3
2	0	—	+	—	++	3		+	--	—	—	1		0	2
	24	—	++	—	+	3		±	++	—	++	3		24	
	40	—	+	—	—		IV	±	++	—	+	3-2 (recov.)	III	40	
PMN															PMN
2	0	—	++	—	++	3		—	—	—	++	3		0	2
	40							—	+	—	—		II	40	
	120	—	++	±	—	2 (recov.)	III-IV								
PMOH	Hrs. of exposure													Hrs. of exposure	PMOH
0.025	72	+	+	±	±	3-2 (recov.)	II								
0.05	24	++	+	—	—	2		+	±	±	—	2-1 (recov.)	I	24	0.05
	96	±	++	±	±	3-2 (recov.)	II-III	++	—	±	—	1	I	96	
0.11	24	++	++	+	—	2	II-III								
0.15	120	+	+	—	±	3-2 (recov.)	II-III								
0.25	24							±	+	—	±	2-3	II-III	24	0.25
0.33	24	—	++	—	±	3	III-IV								
PMN															PMN
0.05	11	+	+	±	±	2-3	II								
	24	++	++	+	±	3-2 (recov.)	II								
0.1	2	+	+	—	+	2-3									0.1
	5	+	+	+	±	"									
	24	—	++	—	++	3	III	+	±	—	—	1-2	I-II	24	0.1
	120							±	—	—	±	3-2	I-II	120	
0.2	24	—	++	—	+	3	III-IV								
0.33	6	—	+	—	++	3									
	72	—	++	±	±	3-2 (recov.)	III-IV								

reaction. Scattered groups of chromosomes which result from the inefficient spindle may enter Grade 3 to give several star metaphase groups and produce a multipolar effect even though spindle formation be absent.) In recovery nuclei enter Grade 2 from Grade 3, and Grade 1 from Grade 2 (Table II).

Grade 3—first phase: spindle completely lacking in c-mitosis (the colchicine-type mitosis or stathmokinesis) with or without accumulation of blocked metaphases or x-form diplo-chromosomes; polyploidization proceeds in non-spindle divisions; second phase: mitotis-free period as after x-rays (Gustafsson 1947); the resting nuclei may subsequently recover to enter c-mitosis first and then normal mitosis (Grades 2 and 1) in diploid and polyploid cells; third phase: resting nuclei undergo occasional endo-mitosis or enter differentiation stage or become pyknotic (Table III).

Grade 4—Premortal changes and death; chromatin agglutinates and clumps in cells caught in mitosis; cells entering division show prometaphase chromosomes that are slender, under-condensed, unflexed, with prominent median heterochromatic knots. Resting nuclei show

TABLE III

CYTOLOGICAL REACTIONS TO ORGANIC MERCURIALS IN ROOT TIPS AT VARIOUS INTERVALS AFTER EXPOSURE FOR AN HOUR IN CINCINNATI TAP WATER

Poison in ppm	Hrs. after expos.	2n Divs.	Over 2 nucleoli	Polypl. Divs.	c-mitosis	Final Gross Reactn.	Remarks
PMOH							
1*	0	—	+	—	+		Rare late 2n telophase.
3.4 ×	20	—	++	—	++		
10^{-6}M	72	—	—	—	—	III-II	No nucleoli; nuclei entering prophase; growth in length already resumed.
2*	0	—	+	—	+		
	20	—	++	—	+		c-mitosis in 2n & 4n nuclei
	72	—	—	—	—	IV	Pyknosis; no gross growth.
PMN							
2	0	—	+	—	++	III-IV	Rare late 2n telophase
3.2 ×	5	—	+	—	++		Full c-mitosis
10^{-6}M	20	++	++	+	+		Spindle recovery in some 2n & 4n
	72	±	+	±	±		Decreased mitotic rate with onset of differentiation.

*Two sets of roots on same bulb treated at same time.

large turgid nucleoli, or none, and become pyknotic. Corresponds to "effective mitotic poisoning" (O'Connor 1950a) see Table VI.

TABLE IV
CYTOLOGICAL REACTIONS DURING CONTINUOUS EXPOSURE TO PMOH IN ROOT TIPS IN VARIOUS TYPES OF WATER.

PMOH ppm	Hrs. of exposure	Solvent	2n D'vs.	over 2 nucleoli	Polypl. Divns.	c-mitosis	Final Gross React.	Remarks
0.1	24	W. W. Filt. Effilt.*	+	+	+	+	III	Anaphase bridges, micro-nuclei, restitution nuclei, intercellular chromatin, karyorhexis.
0.2	168	Raw Ohio River	++	—	±	—	II	Heterochromaty, persistant nucleoli in divn.
0.3	24	Dist. W.	±	++	—	±	III	Anaphase bridges, multipolar groups, tilted spindles, micronuclei, dumbbell nucleoli.
0.33	24	" "	+	++	±	±		
0.5	48	" "	±	++	±	—	III-IV	Heterochromaty, fragments, anaphase bridges, micronuclei, intercellular chromatin.

*Cincinnati Water Works filter effluent contained less than 0.4 ppm combined chlorine, similar to Final Effluent or tap water.

Other Abnormalities Present—(See Tables III to VIII).

Bridges of Chromatin at ana-telophase due to stickiness, or in recovery phase to dicentric chromosomes (Hayden and Smith 1949).

Eroded Chromosomes with multiple secondary constrictions. (Found only rarely in late prophase and in anaphase nuclei (Table VI, PMOH 0.05 ppm Palm Beach water) was reported in roots on old bulbs (Macfarlane et al. 1951). It has been seen in roots on bulbs less than six months old during continuous exposure to sub-narcotic concentrations in both tap waters and in distilled water, but only in the peripheral dermatogen and root cap cells. These tissues were more affected by prolonged treatment than the inner meristem.)

Fragments—usually large and resembled whole chromosome arms. In full Grade 3, c-mitotic reaction, chromosome fragments were absent. Levan (1949) has reported that c-mitosis excludes "radio-mimetic effects". Intercellular chromatin was a common feature of Grade 2 and it is concluded that fragments and micronuclei drifted to the surface of the protoplast and were extruded. In clubbed tips recovering

TABLE V

CYTOLOGICAL REACTION IN ROOTS RECOVERING IN CINCINNATI TAP WATER AFTER VARIOUS TREATMENTS

Treatment & time given, in hrs.	Time after treat. Hrs.	2n Divs.	over 2 nucleoli	Polypl. Divs.	c-mitosis	Remarks
PMOH 1.3 ppm/24	120	+	++	+	—	Fragments* in resting nuclei
CO_2 sat. atmos./24	0	—	++	—	—	*Reactions in indiv. rts.*
	5	+	+	±	—	(Recovery grade 2
		—	+	—	+	(" " 3
		—	+	—	—	(Still resting
	29	+	+	±	—	
Ringers phos. x 1.7 isotonic/2	5	+	++	±	+	Recovery grades 3-2

*0.5 cm. tip of meristem only immersed on 3 cm. long roots. All roots 4 cm. and longer became flaccid in 24 hrs. of exposure.

slow growth in Grade IIIr whole ranks of cells with pyknotic or with "ghost" nuclei have been observed. It is not known whether these are related to the deficiencies caused by extrusion of chromatic material, or whether they represent mosaics of lethals as after chemical mutagens (Auerbach 1951). PMOH was found to be a weak mutagen for plant shoots (Macfarlane 1950a).

Inter-cellular Extruded Chromatin in peripheral cells especially with Cincinnati tap water.

Micronuclei arising from lagging chromosomes and non-centromeric fragments.

Nucleoli increased over normal two, or persisting on mitotic spindle, among or above the chromosomes. Latter also observed in recovery phase of Grade 1.

Ring Chromosomes—unicentric lagging rings observed after a day in PMOH 0.33 ppm in DW. Dicentric rings were observed rarely after same treatment in Cincinnati water. These arose from diplo-chromosome of c-mitosis by adhesion of sticky telomeres at both ends of sister chromatids after division of centromere. (Origin of unicentrics seemed to be in union of sticky telomeres of one chromatid. Reunion of broken chromatids was apparently unusual in these experiments as in Allium generally (Auerbach 1951).)

TABLE VI

CYTOLOGICAL ABNORMALITIES IN ROOT TIPS AFTER VARIOUS TREATMENTS WITH PMOH AND PMN IN CINCINNATI (C) AND PALM BEACH (PB) TAP WATERS.

Water	Poison in ppm	Hrs. after 1 hr. treat.	Clumped mitosis	c-mitosis norm.	c-mitosis clump.	Chrom. bridges	Frag-ments	micro-nucl.	Inter-cellu. chro-matin	Other abnor-malities
	PMOH									
C	10	0	√	√		√			√	2, 5.
		5	√	√	√	√				3, 5.**
C	2	0		√						1
		24		√	√			√	√	4, 8.*
		72				√		√	√	
C	1	0		√		√		√		
		18		√		√		√	√	8*†
C	0.5	24				√	√			4
PB	10	0	√	√	√	√				2, 4.**
PB	2	0		√		√				1, 3, 4,
		40		√						2, 3, 4.
	1.3	24				√	√			2
		Hrs. of exposure								
Dist. W.	0.33	24		√		√			√	1, 5, 6, 7.
C	"	24		√		√		√	√	2, 6, 7 T.
C	0.1	24				√	√	√	√	5
C	0.05	96		√		√		√	√	2, 7 T.
PB	1	30	√	√	√					9**
PB	0.25	24		√		√				2. 3.
"	0.05	24				√	√			1, 5.
	PMN									
	0.1	24				√	√	√	√	1, 7 T.
		120						√		1, 2.

*Few 4-nucleate cells produced by "somatic reduction".
†Up to 8 nucleoli in some resting nuclei.
**Effective mitotic doses for 1 hr. in C and PB and for 24 hrs. in PB water.

Key to column 10: 1 Tilted spindle, 2 arrest in prophase, 3 multipolar spindle, 4 prominent heterochromatic regions, 5 eroded chromosomes (extra constrictions), 6 Unicentric ring chromosome, 7 dicentric ring chromosome, 8 general pyknosis, T in telophase.

Spindle Tilted diagonally in the cell (Östergren 1950), flat topped and multi-polar spindles.

Under-Condensed, Unflexed Prophase and Metaphase Chromosomes with reduced stainability and prominent median heterochromatic knots; this abnormality persists in recovery stages.

Cytological reactions in Grades 1 and 2 occurred in sub-narcotic concentrations. Reversal and recovery to normal were not prevented

TABLE VII

CYTOLOGICAL ANALYSIS OF ROOTS 3-4 CM. LONG GROWN AND TREATED IN PALM BEACH WATER 24 HOURS AFTER CONTINUOUS EXPOSURE TO EQUIMOLAR CONCENTRATIONS. ($1.6 - 1.7 \times 10^{-7}$M).

Treatments run simultaneously. Tips fixed 5 pm. Reactions: Gross Grade I, Cytological Grade 1.

Bulb.	Treatment	Total cells.	Total divs.	No. cells in P.	M.	A.	T.	Mitotic Index %	M/P Ratio	Av. growth cm./day
a	Control	650	20	7	5	3	5	3	0.7	1.5
b	PMN 0.1 ppm	646	29	14	3	5	7	4.5	0.2	1.1
c	PMOH 0.05 ppm	774	47	22	8	14	6	6	0.36	1.1

Abnormalities

(a) In 2 tips: 1 tilted spindle; 1 lagging chromosome; 1 Ana. 4n; rarely 3 nucleoli.
(b) In 1 tip; 2 tilted spindles; 2 micro-nuclei; 1 fragment (chros. arm).
(c) In 1 tip; 1 tilted spindle; 1 Ana. 4n; 1 reconstituted 4n nucleus; 4 nucleoli in 4 resting nuclei among 1,000.

by structural changes and polyploidization (abnormal cells were eventually outgrown by unaffected nuclei), but by the onset of differentiation in the meristem shown by peripherally located nuclei (Vanderlyn 1948) and vermiform nuclei.

In all these experiments (except after PMOH 0.5 ppm for an hour) the dividing cells coming into division attained, eventually, a Grade 3 c-mitotic reaction before entering a mitotic-free period. (Tables II to IV). In recovery the cells might again go through a c-mitotic and a slow spindle stage (Grade 2) as indicated in Table II. The Grade 2 reaction shown in Table IV after 2 days in PMOH 0.5 ppm in distilled water was judged to be a recovery phase although the tissue eventually died as a consequence of the destruction of the older tissue above. Region 3 was already flaccid and water logged when the tips were fixed.

In recovery among 2n and 4n nuclei, diploids tended to come into division first (Table II) in accordance with their known greater metabolic growth energy.

Influence of Solvent on Cytological Reactions.

The cytological reactions of the meristematic cells at various intervals after an hour's exposure to PMOH and PMN, and also during continuous treatment, with Cincinnati and Palm Beach tap waters as solvents, are shown in Table II. The final gross reaction to each treatment is also given. The deleterious effect of Cincinnati water, which is demonstrated in Figure 1, is also apparent here. With the short treatment in PMOH 2 ppm (6.8×10^{-6}M) in this water spindle

TABLE VIII

CYTOLOGICAL ANALYSIS OF MERISTEMS OF ROOTS 4-7 CM. LONG AFTER 24 HOURS IN DISTILLED WATER (DW) OR IN CINCINNATI WATERWORKS FINAL EFFLUENT (CFE) WITH PMOH IN CONCENTRATION CAUSING: GROSS REACTION GRADE IIIs (static), CYTOLOGICAL REACTION GRADE 2-3.

Average growth in 24 Hours of treatment 1.5 mm. *N.B.* Roots of Bulb *a* were cultured in DW and of bulb *b* in CFE from start.

Bulb	PMOH ppm Treatment	Cells Total	Divs. Total	Mit. Index	Diploid Divs. P	M	A	T	Tetrapl. Divs. P	M	A	T	Polypl. r. n.	Divs. with Fragments	Cytol. Grade
a	0.33 in DW	1124	51	4.5	5	9	4	19	9	—	4	1	100	11	2
b	0.1 in CFE(1)	1046	101	9.7	9	36+	—	—	38	7+	11+	—	246	0	3
	(2)	881	92	6.1	10	28+	—	3	9	1	3	—	—	—	3

+c-mitotic figures.

Abnormalities

(a) Micronuclei 196; intercellular chromatin patches 104; Total fragments 22; lagging ring chros. 3; multinucleate resting cells 98; chromosome bridges 2; tilted spindles 2.

(b) Root 1. Micronuclei 210; inter cellular chromatin patches ++; binucleate cells 2; multipolar groups 3-4; Karyorhexis ++. Dividing cells in full c-mitosis. Root 2. Similar but early c-mitosis. Multipolar late telophase 1.

formation was suppressed and c-mitosis present at the end of the hour and all division had ceased the second day after exposure. Loss of nucleoli and finally pyknosis followed, while the cells above in region 3 became flaccid and necrotic. In Palm Beach water, however, normal mitosis continued throughout the hour of treatment and the cells entered Grade 3 (c-mitosis) in the ensuing day; some spindle mitosis reappeared in recovery of Grade 2 reaction after 40 hours in tap water. With similar continuous treatments the recovery of normal mitosis in adaptation was delayed when Cincinnati water was the solvent as compared to Palm Beach water (Table II).

A lower sensitivity of the dividing cells in distilled water than in Cincinnati tap water is demonstrated in Table IV by the presence of some spindle mitosis in 2n and 4n nuclei after 24 hours in PMOH 0.33 ppm in DW, when at the same time the gross increment was only 1 to 2 mm. The same condition is shown in Table VIII.

Equivalent Cytological Reactions With Short and Long Treatments

Examples of parallel cytological reactions in 24 hours following after an hour's treatment and after continuous exposure in the same tap water are shown in Table II. In Cincinnati water PMOH 2 ppm and 0.33 ppm gave full Grade 3 (c-mitosis) and the tissues contained some lobed restitution 4n nuclei, multinucleate cells, micro-nuclei and intercellular chromatin (Table VI), the results of an earlier Grade 2 reaction. The estimated penetration in an hour of the PMOH 2 ppm into the meristem is therefore 16.5% to give an internal concentration of 0.33 ppm. The final gross reaction was Grade IV, or death, in all roots over 3 cm. long and the estimated penetration into region 3 was 23% (Figure 1).

Also in Cincinnati water parallel cytological reactions were found one day after an hour's exposure to PMOH 0.5 ppm and after a day in 0.1 ppm. This suggests a 20% penetration, or the same proportion that was found for region 3 for an hour in the same low concentration (Figure 1). PMOH 0.1 ppm. was the lower threshold for Grade II in this solvent. In Palm Beach water twenty-four hours after an hour in PMOH 1.3 ppm the dividing cells were recovering Grade 2 after c-mitosis. After a day continuously in PMOH 0.25 ppm they were entering Grade 3 from Grade 2, which is a weaker reaction. Therefore a penetration of over 20% may be deduced for region 1 for 1.3 ppm in an hour. Judged from the gross reactions (Grades II and III) the

penetration of 1.3 ppm into region 3 in an hour was 20% (Figure 1). It thus appears as if there may be a proportionately greater penetration into region 1 in concentrations which do not seriously affect the elongating cells of region 3.

Because cytological reactions in sub-lethal concentrations are quickly reversible in recovery phases it is more difficult to judge similarity of effects microscopically than it is macroscopically. (When there was a final differential reaction between members of the same set of roots, it was not possible to know the final gross reaction of an individual root examined cytologically in 24 hours or less).

With Grade 2-3 reactions in Cincinnati water there were noticeably more micro-nuclei and intercellular chromatin patches in region 1 than in the same grades of reaction in Palm Beach water (Table VI). These effects were enhanced by traces of chlorine, both free and bound, as also were polyploidization and the accumulation of blocked metaphases in Grade 3. The latter is shown in Table IV after 24 hours in PMOH 0.1 ppm in Cincinnati Water Works filter effluent which contained 0.35 ppm free chlorine and 0.44 ppm bound chlorine when collected. Gross reactions were also stepped up in river water near the lethal threshold of PMOH by traces of chlorine. (Macfarlane et al. 1951). Roots recovered both grossly and cytologically in continuous exposure to PMOH 0.2 ppm in river water (Table IV).

In Cincinnati water during 24 hour's exposure there was a slightly stronger cytological reaction to PMN 0.1 ppm (earlier and longer c-reaction) than to equimolar PMOH 0.05 ppm (Table II) and slightly greater prophase arrest (Table VII). PMN 0.1 ppm produced a full c-mitotic reaction in twenty-four hours in Cincinnati water and only a Grades 1 and 2 reaction in the same period in Palm Beach water (Table II).

Effective Mitotic Concentration and Influence of Solvent

In the Grade 4 type of cytological reaction the chromosomes of cells in division stick together or clump. The amount of poison which produces this reaction in embryonic cells has been called "the effective mitotic concentration or EMC" by O'Connor (1950a). This was reached during an hour in PMOH 10 ppm in Cincinnati water, and subsequently region 3 cells lost turgor rapidly and were water-logged in under 12 hours. This concentration was five times the MLD for one hour's treatment for region 3 cells. That all region 1 cells were not

killed immediately by the PMOH 10 ppm was shown by the fact that roots fixed at the end of an hour's exposure contained: (1) extra (3-4) nucleoli in many resting nuclei; (2) some nuclei in early c-mitotic (non-spindle) metaphase, the members of the pairs of sister chromatids lying close to each other; (3) an occasional late telophase sometimes with a chromatin bridge; (4) rarely an early anaphase without external spindle in which the two groups of daughter chromosomes seemed to be separated by an ovate mass of material on the equator; (5) lobed restitution nuclei and "multipolar" groups of chromosomes, which indicated that the cells had passed through a Grade 2 reaction in the poison. Furthermore root tips fixed 5 hours after the treatment showed an increase in blocked metaphases over those fixed at the zero hour; some clumped blocked metaphases were also observed (Table VI).

After an hour in PMOH 10 ppm in Palm Beach water a few clumped spindle metaphases and also normal and clumped blocked metaphases were observed (Table VI).

A day after an hour in PMOH 2 ppm in Cincinnati water some clumped c-mitotic metaphases appeared among cells in full Grade 3 reaction (Table VI). Either the poison continued to accumulate in these cells after the treatment, or the effects during the hour were irreversible and proceeded towards death of the cells. After similar treatment in Palm Beach water there were no clumped metaphases and all cells were in resting condition at the end of 24 hours in water. Recovery to full c-mitosis occurred during the second day of treatment.

Reactions of Dividing Cells as Affected by Concentration and to Sensitivity of Region 3

The extreme sensitivity of the meristem, region 1, to slight differences in concentration of these poisons is brought out in Table III which shows the reactions in two sets of roots on the same bulb which were exposed for 1 hour to PMOH 1 ppm and 2 ppm respectively in Cincinnati water. Grade 3 was reached in both concentrations at the end of the hour. In the roots exposed to 2 ppm on the following day the cells of region 3 were already moribund so that the supply of water and solutes from above for the meristem was cut off. On the third day after treatment these meristematic cells were all resting, and some were pyknotic, while karyorhexis was prominent in squash preparations. At the same time in the other set of root tips, although the meristematic

cells also were all resting, many were entering prophase. Grossly they had already started to elongate at an average rate of 5 mm. a day after a 48 hour static period. Cytologically the first day after exposure the two sets of meristems differed only in degree of reaction. Therefore it may be inferred that the death of the dividing cells after PMOH 2 ppm was at least partly a consequence of the death of the older cells.

In Grade I reactions a slight initial stimulation of the rate of division occurred in some cases while growth in length was below the control rate (Table VII). This could be due to (a) differential tissue sensitivity; (b) slower reactivity in dividing cells; or (c) delayed penetration of the poison to them. Similarly, a day later, gross increment rate was increased over control in some Grade I reactions while mitotic rate remained low.

Some roots after 24 hours' exposure to concentrations of PMOH in DW which produced immediate gross arrest (Grade III) showed a normal mitotic index (Table VIII) with only partial, or even no suppression of spindle formation (see also Table II, Macfarlane et al. 1951). This again demonstrates the independent, or non-synchronous, reactions of the dividing and elongating cells in each root.

That the time factor is less important in relation to an irreversible lethal reaction in region 1 than it is in regions 2-3 was shown by the recovery of normal mitosis in Grade III clubbed tips which had been in Cincinnati water for five days after an exposure of the tip 0.5 cm. only on 3 cm. long roots, to 1.3 ppm PMOH (Table V) for 24 hours. Some dividing cells showed one or two large chromosome fragments. This concentration of poison was more than twice the lethal dose for regions 2 and 3 tissues in continuous exposure, yet when the youngest tissue only was exposed, the cells survived in it for a day and were able to recover. It seems therefore, that the upper limit for an arresting or narcotic dose for the dividing cells may actually be nearly the same for an hour and for prolongued exposure or around 1.5 ppm PMOH (5.2×10^{-6}M) in Cincinnati water.

Figure 1 suggests that penetration into regions 2 and 3 is lowest at PMOH 1.5 ppm in Cincinnati water in an hour's exposure. This concentration may perhaps arrest vital functions in the sensitive enlarging cells first and as a consequence the supply of solutes to the central tissue of region 1 will be cut down. In longer exposures an irreversible denaturation of the proteins of the enlarging cells may occur (Seibert et al. 1950) while the cells below are merely narcotized and enter a state of anoxia.

Comparative Reactions After CO_2 and Hypertonic Ringer's Treatment

It is shown in Table V that in recovery from 24 hours' exposure to a moist atmosphere of CO_2, cells of region 1 entered Grade 3 (c-mitosis) and Grade 2 to 1 cytological reactions after 5 hours in Cincinnati water. A similar 5 hour recovery reaction was observed after 2 hours in slightly hypertonic Ringer's phosphate solution (1.7 times normal tonicity). In both experiments the proportion of normal and c-mitotic divisions varied considerably from root to root. A day after the CO_2 treatment in some roots all the cells of region 1 were still in resting condition, others showed a few non-spindle metaphases only, while in a few roots there was normal mitosis with a very low rate of division. After a further day in water all CO_2 treated roots showed normal mitosis in 2n and 4n nuclei with a slightly reduced mitotic index. During this day the roots had grown 3-4 mm. beyond tiny c-tumors. Resting nuclei contained 1-3 large nucleoli and rarely a tilted telophase figure was observed (Östergren 1950).

Immediately after 24 hours in CO_2 all region 1 cells were in resting state and many showed three large turgid nucleoli. This feature gradually became less pronounced upon return to water. Extra nucleoli were also present after 2 hours in the hypertonic Ringer's.

Nucleolar Changes in Mercurial Poisoning

The presence of extra nucleoli was observed after treatment with the mercurials as shown in Tables II to V and VII. An increase in the number of nucleoli can result from multiplication of the nucleolus organizing chromosomes and has been used as diagnostic of polyploidy. In arrested c-tumors (Grade IIIs) when nuclei have undergone several c-mitotic, spindleless divisions, six and ten small nucleoli have been observed e.g., after 72 hours in PMN 0.33 in Cincinnati water (Table II). A high number of nucleoli was also found in some resting nuclei one day after an hour in PMOH 1 ppm (Table VI) and have been recorded after colchicine treatment by Levan (1938).

There is another possible explanation for the presence of one or two extra nucleoli in onion root cells. Some varieties of *Allium Cepa* have been reported by Mensinkai (1939a and b) to be secondarily balanced diploids with two pairs of nucleolar chromosomes, with a diploid complement of sixteen, containing two pairs of non-homologous nucleolar organizers. A total of four nucleoli could therefore appear in a diploid resting nucleus. Normally these tend to coalesce into one or

two coascervates and I suggest that this may be inhibited by increased viscosity of the plasmasome nucleic acid and also by impeded chromosome movements in narcotized young daughter nuclei.

Root tips fixed after 24 hours in PMOH 0.33 ppm in DW (Table IV) showed a few dumbbell shaped nucleoli in resting cells suggesting a slow or incomplete coalescence of two highly viscid drops. One trinucleate cell had a dumbbell shaped nucleolus in the largest nucleus and one globular nucleolus in each of the two smaller nuclei. It has been observed several times that in Grade 2 reactions the nucleolar organizer chromosomes reached the poles first, and sometimes alone, to form two micronuclei each with a nucleolus. In the latter condition the rest of the chromosomes lag behind on the equator and eventually from a restitution aneuploid nucleus there. Cytoplasmic streaming appears to displace such micro-nuclei, and any separated groups of one or two chromosomes, and to carry them to the periphery of the cell.

Another factor in these cytological aberrations may be a depression of the activity of the depolymerase which interferes with the formation of DNA from RNA in nucleic acid metabolism. This condition has been reported in precancerous liver tissue in rat (Cantero et al. 1950). Dustin (1947) concluded that the nucleolus is more resistant than other cellular structures.

Discussion

Non-specific Character of Cytological Reactions

These organic mercurials are known to reduce oxygen uptake in cells of yeast and vertebrate tissue slices (Cook et al. 1946) and also to inhibit enzymes of the respiratory chain, including some dependent upon the —SH group. Should similar effects occur in the root tip in the absorbing cells above the meristem, this would lower their suction pressure to produce increased concentration of cell sap and of protoplasmic viscosity. Artificial drought conditions were shown by Beck (1942) to inhibit enzymes needed for chlorophyll production. With reduced hydration there will be an accumulation of CO_2 and of lactic acid to cause arrest of spindle formation (Thomas 1945).

An amitotic phase was produced in my experiments by 24 hours' exposure to a moist atmosphere of CO_2 and after exposure to the mercurials. The same phenomenon resulted in Allium roots from subnarcotic and narcotic treatments with colchicine (Levan 1938) and after x-radiation (Sax 1941). Chromosome fragmentation which

occurred after x-ray treatment (Sax 1941) also appeared in a low degree after CO_2 exposure and after mercury treatments. Grades 3 and 2 which appeared in recovery phase after CO_2, hypertonic Ringer's phosphate and PMOH, were observed after cold treatment (Huskins and Chen 1950). It is obvious that the same cytological symptoms arise from many different causes (MacCardle 1951) and these experiments have not revealed any specific effects attributable to the mercurials themselves.

Colchicine reduces cytoplasmic viscosity (Northen 1951) and contracts the protein chains in the spindle (Ehrenberg 1946). The mercurials have a similar effect on the spindle and since gelation of cytoplasm is needed for chromosome movements (Marsland 1951) viscosity would appear to be reduced. This has not been verified directly but there is some evidence for a reversible reduced permeability of treated roots (Figure 1). Since permeability of the protoplast usually varies directly with viscosity (Goldforb 1935), we may deduce a reduced viscosity followed by a premortal gelation as permeability rises again. N_2 and CO_2 treatments were shown by Hoagland and Broyer (1942) to cause an initial decrease in root permeability, followed by an increase concomitant with protoplasmic breakdown. The premortal increase in permeability of cells to poisons corresponds to a gradual irreversible unfolding, or denaturation of the polypeptide chains. Seibert, Kreke and Cook, (1950) reported that the organic mercurials act similarly to heat in causing denaturation of enzyme proteins. Like heat inactivation this process depending on degree, may also be reversible (McElroy 1947). Because these poisons depress oxygen uptake, they may be expected to have similar effects to anoxia, in sub-lethal concentrations and to inhibit cell enlargement which is highly aerobic (Thimann 1949).

Oxygen lack could also be a direct cause of the ageing effects of treatment with the organic mercurials which are manifested in precocious differentiation of the embryonic cells of the root tips. Rosene (1950) found that the short lived root hairs rapidly lost their maximum water absorbing power as they aged. Berry (1949) demonstrated that the metabolic rates of the second and third 5 mm. regions in the onion root tip (my region 3) were lower than that of the terminal 5 mm. The latter includes both the dividing cells and the enlarging cells (my regions 1 and 2). With a reduced oxygen supply differentiation apparently can proceed but not the primary forms of growth.

The recorded effect of the water used as solvent may be due to the influence of differences in ionic balance on permeability (Macfarlane et al. 1951).

Path of Penetration into Meristem

Roots exposed to lethal and sub-lethal concentrations of the organic mercurials showed a lag in toxic reactions in region 1 (meristem) as compared with regions 2 and 3. In soil cultures solutes usually reach the meristem from the root hair region above it, at the end of the youngest xylem vessels (Rosene 1950). Root hairs fail to develop on onions in water culture, but it is not improbable that the same path is followed by the bulk of the water absorbed as in soil. Therefore the effect of any substance on the older cells may well be a major factor in the rate of its penetration to the meristem. With colchicine and onion root tips Levan (1938) found that the degree of polyploidization was greatest in the proximal region and less at the distal end of the meristem. Levan and Lotfy (1949) reported that this was true with naphthalene acetic acid also. My preparations showed the same condition, except in the vestigial root-cap and the peripheral dermatogen cells, which possibly obtained poison by direct exchange with the medium.

It has been noted above that there may be a proportionately greater penetration of poison into region 1 when the absorbing cells above are not seriously incapacitated. Another example of this is given in Table VII with subnarcotic concentrations of PMOH and PMN which gave gross Grade I. They caused an arrest in prophase along with heterochromaty, chromatin bridges, a low rate of chromosome fragmentation as well as super-numerary and persistant nucleoli.

Enzyme Systems Inactivation by Organic Mercurials

Kreke, Kroger and Cook (1949) found that with animal tissue *in vitro* in Ringer's solution, the concentration of organic mercurial which gave 50% depression of the succinoxidase system in 1-2 hours was PMN 3.6 x 10^{-6}M and PMOH 5.0 x 10^{-6}M. These concentrations are of the same order as those I have found to be the upper limit for Grade II gross reactions in onion root tips for an hour's exposure i.e., arrest with c-tumor formation followed by normal recovery,—namely PMN 1.6 x 10^{-6}M (1 ppm) and PMOH 5.2 x 10^{-6}M (1.5 ppm) see Tables I and III. (Phenylmercuric chloride 7 x 10^{-7}M caused a 50% inhibition of growth by cell enlargement in Pisum shoots in a day's exposure

(Thimann 1949). For Grade II the upper limit of concentration in prolonged exposure was PMOH 0.15 ppm in Cincinnati tap water and in distilled water or 5.2 x 10^{-7}M, and 0.25 ppm or 8.5 x 10^{-7}M in Palm Beach water).

Kreke, Kroger and Cook also found that the succinic dehydrogenase system was less sensitive than the succinoxidase and needed PMN 3.2 x 10^{-5}M or PMOH 7.5 x 10^{-5}M to produce a 50% depression in 1 to 2 hours' exposure. These concentrations are close to the lowest effective mitotic concentration that gave clumping of chromosomes in dividing cells in an hour namely PMOH 3.4 x 10^{-5}M, in both Cincinnati and Palm Beach waters. (The shoots of Elodea and Ruppia in Palm Beach water were less sensitive to PMOH in short exposure than Allium roots and the MLD for an hour was 6.5 x 10^{-5}M. (Unpublished data.)

These data indicate that growth by cell enlargement and also spindle formation, both of which take place in the cytoplasm, are dependent upon the aerobic succinoxidase enzyme system. Further they suggest that the nucleus can go into spindleless division (c-mitosis) under anaerobic conditions while the dehydrogenases are able to function. O'Connor (1950b.) used other respiratory poisons which, like the mercurials, combined with —SH groups and found that in chick embryo tissue *in vitro* mitotic damage began in the same concentrations in which aerobic glycolysis started to be depressed. The enhanced sensitivity of onion roots in continuous exposure is attributable to the effects induced in the cytoplasm by anoxia and reduced hydration. The two phases of growth, which are organized in two tissues in the root tip differ in their sensitivity to the inhibiting effects of the mercurials. Effective polyploidogeni. power depends upon a low toxicity for the absorbing cells and the enlarging cells. The cytoplasm of these older cells may have less resistance to poisoning.

The c-tumor Reaction in Bioassay

It was easier to correlate parallel gross reactions than parallel cytological reactions in different treatments of the roots in attempting to assay the penetration of poisons under various conditions (Macfarlane 1950b) and also in antagonism work (Macfarlane and Nadeau 1948).

In all treatments which produced gross and cytological effects the cells of region 2, immediately above the dividing cells, became swollen with hydropic cytoplasm. This seems to result from a metabolic shock

which is intensified in some solvents and may be related to a decrease in cytoplasmic viscosity in which the protoplasmic proteins were disaggregated (Northen 1951). During continuous treatment with high dilutions of PMN and PMOH with Grades I and II gross effects, a small c-tumor reaction was produced each time the solution was renewed (Macfarlane and Schmock 1948). Since orientation of the cell wall micellae depends upon that of the surface layers of the cytoplasm (Frey-Wyssling 1948, Preston, 1939) the young cells are probably irrevocably deformed before they start to enlarge. Unlike sub-lethal cytological reactions, the c-tumor was an irreversible one and varied in intensity with concentration of the mercurial.

Recovery of mitotic spindle formation occurred within 24 hours after an hour's exposure to concentrations of the mercurials which were 50% lethal for the older tissue (Grades III-IV) see Tables IV and V. Therefore the upper threshold concentrations for recovery of structural viscosity of the spindle were higher than those for recovery of growth by cell enlargement, provided, of course, that the cells of region 1 received as much of the poison as the cells of regions 2 and 3.

Summary

1. Gross reactions of onion root tips to organic mercurials can be duplicated in continuous and one-hour treatments. The ratio between any two such concentrations seems to indicate the amount of poison which penetrated the tissue in the short exposure. At the maximum concentration which permitted normal recovery after c-tumor formation and temporary arrest (Grade II) the proportion of penetration was lowest, falling to around 10% in tap and in river water. This concentration was PMOH 5 x 10^{-6}M for an hour in Cincinnati tap water and in Palm Beach water PMOH 8.5 x 10^{-6}M. In DW permanent gross arrest (Grade III) was caused by an hour in PMOH 5.2 x 10^{-6}M, but permeability continued to fall up to 1.1 x 10^{-5}M before the premortal increase in permeability was initiated.

2. Cytological reactions have been classified into four categories and the observed abnormalities described. Direct and cumulative effects led to c-mitosis in Grade 3. The cells then entered an amitotic phase and during recovery the whole series of reactions from c-mitosis through inefficient spindle divisions (Grade 2) to a slowing of prophase events (Grade 1) was repeated in reverse. This made it difficult to correlate similar cytological reactions in short and long exposure.

3. Full c-mitosis with accumulation of blocked metaphases occurred during an hour in PMOH 3.4 to 6.8 x 10^{-6}M in Cincinnati water, but did not appear until some hours later after similar treatment in Palm Beach water. Similar effects after exposure to CO_2 and slightly hypertonic Ringer's solution suggest that anoxia and lack of hydration may be involved in the effects of organic mercurials.

4. The meristematic tissue was less sensitive than the elongating and absorbing regions. The effective mitotic concentration for clumping in an hours' exposure was phenylmercuric hydroxide 10 ppm (3.4 x 10^{-5}M) in Cincinnati water. This was five times the MLD for older tissue in the penultimate 1 cm. of the tip; it was about half the concentration which produced a 50% depression of succinic dehydragenase in animal tissue *in vitro*.

5. No specific reaction attributable to the mercury ion has been found. The gross reactions provided a delicate test for differences in concentration of the poisons and may be useful in bioassay.

6. The differences in permeability which were detected and their possible relationship to viscosity changes in the protoplasm are discussed.

7. Data suggest that even in water culture the solutes reach the root meristem chiefly from above and that a temporary poisoning of the succinoxidase system of the older cells may automatically protect the dividing cells.

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A TOLERANCE THEORY OF VITAMIN ACTION[1]

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I. PRESENT CONCEPTS OF VITAMIN ACTION

When Funk in 1912 coined the term vitamin, he assumed these compounds to be nitrogenous substances indispensible as building stones for some vital cell constituents, just as some amino acids are known to be indispensible building blocks for protoplasm. When a further study revealed that some of the vitamins were coenzymes, Eakin (1950) stated:

> "It seems reasonable to offer as a general hypothesis that the only function of the B vitamins *per se* is to participate directly in certain specific enzymatic reactions."

Besides these two viewpoints, we have the general, vague statement that vitamins are "accessory growth factors," or more precisely, in Gould's Medical Dictionary (1949), that they are:

> "Required for the normal growth and maintenance of life of animals, including man who, as a rule, are unable to synthesize these compounds. They are effective in small amounts, and do not furnish energy, but are essential for transformation of energy and for the regulation of the metabolism in the organism."

The shift of emphasis from building blocks of the protoplasm to the furnishing of energy is not necessarily contradictory since growth requires a source of energy, and energy liberation from food is brought about by a quite complex mechanism built *by* the cell and ***within*** the cell. But the role of the vitamins in the living cell has remained rather vague from the viewpoint of the biochemist, though the chemical structure of most vitamins is known now, and some of them are synthesized commercially.

[1]This investigation has been made possible by a grant from the Division of Research Grants, National Institutes of Health, Bethesda, Maryland.

Vitamin Requirements by Bacteria

The complexity of the symptoms of the avitaminoses in man and animals is too confusing to permit a clear understanding of the biochemistry of the role of vitamins. It seems more promising to follow the precedent of Pfeffer (1904) who clarified some problems in plant physiology by working with molds, or of Meyerhof (1914) who studied basic principles of cell metabolism by switching from higher animals to nitrate bacteria. Since a number of bacteria are known to require certain vitamins for their development, the method of studying the basic function of vitamins with the help of microorganisms was tried, and the results obtained with riboflavin may serve as the pattern for a general method to study the function of other vitamins as well. This method has the advantage of simple technique and quick results.

A good deal is known about the role of vitamins in bacteria, thanks largely to the untiring work of R. J. Williams and his associates. Their results made it possible to develop a method of vitamin assay for all water-soluble B vitamins. For instance, *Lactobacillus casei* grows well only if the culture medium contains riboflavin (Vitamin B_2). To measure the riboflavin content of a food, the lactobacillus is cultivated in a nutrient medium free from riboflavin to which are added graded quantities of the food to be tested. Parallel cultures receive graded quantities of the pure vitamin. Equal amounts of bacterial growth (measured by turbidity) or equal amounts of lactic acid produced (measured by titration) indicate equal amounts of riboflavin present. Without riboflavin, the lactobacillus grows poorly, and produces little lactic acid; but with the vitamin, it multiplies to large numbers, and produces much acid, depending upon the amount of vitamin present—(Tables VI and VII). The same principle is used with other B-vitamins (Williams, 1941).

These two phenomena, namely more fermentation products and more cells in the presence of vitamin, are usually explained in textbooks by the statement that riboflavin is a growth factor, and therefore permits the development of unusually large numbers of cells which, in turn, produce more acid, so that the amount of acid formed is a measure for the amount of riboflavin. This seemingly simple explanation has the disadvantage that it is not in agreement with some well-known physiological facts. The statement that more cells produce more products, is contradictory to our knowledge of enzyme reactions, and has been disproved for bacteria thirty years ago. The present paper is

an attempt to explain the biochemical function of riboflavin without contradicting established facts.

The Cause of the Endpoint of Fermentation (Maximal Concentration of Products)

The lactic fermentation is an enzymatic reaction (Buchner and Meisenheimer, 1903). Enzyme reactions with an abundance of substrate come to an end, due to the accumulation of the products of reaction. Urease with an unlimited amount of urea will continue to change the substrate to ammonium carbonate until the alkalinity blocks further hydrolysis. The enzyme is not irreversibly inactivated, or denatured; neutralization of the ammonium carbonate renews the activity of the enzyme.

The same is true for fermentations. Bread yeast will ferment a surplus of sugar to 12% alcohol. If 8% of alcohol is added at the beginning, the yeast produces only 4% more, so that the fermentation is blocked again at 12%. Different varieties of yeast are blocked by different concentrations of alcohol. Similarly, the formation of lactic acid from glucose by *Lactobacillus casei* is blocked by 1.15% lactic acid, regardless of whether the acid is a fermentation product, or whether all or part of it has been added as pure lactic acid. Table 1 brings the experimental proof.

TABLE 1
Total Lactic Acid Produced by *L. casei* in a Medium to Which Lactic Acid Has Been Added

Lactic Acid Added to Medium	0%	0.1%	0.2%	0.3%	0.4%	0.5%
Total Lactic Acid of Culture after 80 hours	1.12%	1.16%	1.17%	1.08%	1.18%	1.15%

This inhibition of a fermentation by a definite concentration of the fermentation product characterizes it as an enzymatic reaction. The final concentration of a fermentation product in any fermentation has been called the "endpoint of fermentation." This endpoint is not an equilibrium of the type known in monomolecular or multimolecular reactions. It is not a function of the concentration of the substrate. If a yeast in grape juice with 25% glucose produces 11% alcohol, the addition of more sugar has no effect upon the alcohol concentration. A lactobacillus will not produce more acid in milk when more lactose is added.

The endpoint of fermentation is characteristic for the species. In the same malt extract, different species of yeast produce different amounts of alcohol. In milk, different species of streptococci and lactobacilli produce different concentrations of lactic acid. These concentrations are characteristic for the species or variety, and are used for identification of the organisms. They are inheritable properties. The endpoint of fermentation must be considered a property of the fermenting enzyme, not of the total cell. The addition of vitamin changes only the tolerance of the enzyme, while the remainder of the cell, including its genetics, remains unaffected, as will be shown later.

Cell Number and Endpoint of Fermentation

The customary explanation of vitamin action upon bacteria is based on the assumption that more cells produce a higher concentration of fermentation products, i.e., a higher endpoint of fermentation. There is no evidence for this, and all experience speaks against it. As has been stated above, urease will change urea to ammonium carbonate until a certain limiting pH is reached. The addition of more urea will not change this endpoint, nor will the addition of more enzyme have any effect. One hundred milligrams of urease are inhibited by the same pH as ten milligrams. This applies also to fermentations. Table 2

TABLE 2

Alcohol and Carbon Dioxide Produced in 100 ml of Glucose Solution by Graded Amounts of Bakers Yeast

Exp.	Grams yeast added	0.02	0.04	0.08	0.16	0.31	0.62	1.25	2.5	5.0	10.0	20.0
I	Alcohol	11.75	lost	11.80	11.75	12.45	lost	lost	12.60	13.35	13.35	
	CO_2	11.04	10.95	11.01	11.04	11.14	11.04	11.56	12.02	12.44	11.91	
II	Alcohol		12.35	12.00	11.95	12.90	13.10	13.02	13.62	13.73	13.90	10.35
	CO_2		10.92	11.03	10.99	11.17	11.38	11.60	12.05	12.63	12.45	11.83

(from Rahn, 1932, p. 147) gives the amounts of alcohol and of carbon dioxide produced in a raisin decoction with plenty of added glucose by different quantities of baker's yeast, the largest amount of yeast being 1,000 times as large as the smallest. The two experiments prove quite conclusively that more cells do not produce a higher endpoint of fermentation. Each cell continues to ferment until 13% of alcohol is accumulated which blocks all enzymic action, regardless of the amount of enzyme, or the number of yeast cells or the amount of sugar present.

TABLE 3
ACID FORMATION IN MILK BY *Streptococcus glycerinaceus* WITH DIFFERENT CELL QUANTITIES

Strain	Number of Cells per ml	Titrated Acidity as % lactic acid, after 120 hours	pH after 144 hours
A	37,000,000	0.59	4.88
	3,705,000,000	0.66	4.69
B	24,000,000	0.62	4.84
	7,310,000,000	0.79	4.59

Table 3 proves the same independence of the endpoint of fermentation from the number of acting cells for the lactic acid production by *Streptococcus glycerinaceus,* (data from Rahn, 1932, p. 148). A very large increase in cells by means of centrifugation did produce a very slight increase in acidity. With Strain A, a hundredfold increase in cells (10,000% increase) gave an 11% increase in acid; with Strain B, a 1,200% increase in cells resulted in 28% more acid.

II. RIBOFLAVIN

Different Behavior of Vitamin-fed Cells. These increases in the final amount of fermentation products are not at all of the order of magnitude commonly observed in vitamin experiments. A typical assay record is given in Table 4 which shows that a doubling of the

TABLE 4
TURBIDITY AND AMOUNT OF LACTIC ACID PRODUCED IN A RIBOFLAVIN-FREE MEDIUM WITH VARIOUS AMOUNTS OF RIBOFLAVIN

Riboflavin added: µg per 10 ml		mg Riboflavin per liter 0	5	10	20	30	50
Turbidity	48 hrs	11	21	32	100	140	205
	72 hrs	11	23	34	91	125	175
	8 days	11	19	35	94	165	220
% Lactic	48 hrs	0	0.19	0.25	0.49	0.57	0.69
Acid	72 hrs	0	0.22	0.23	0.53	0.73	0.82
Produced	8 days	0	0.25	0.34	0.66	0.85	1.19

riboflavin content of the medium from 0.1 to 0.2 mg per liter resulted in an increased turbidity of 300% and in an acid increase of 60 to 70%. Table 5 gives a comparison of the ratios. While an elevenfold increase in turbidity (= 1,100%) caused by vitamin, produces 38% more acid, a 100 fold increase (= 10,000%) by crowding of the cells through centrifugation, produced little or no increase in fermentation products, the maximum increase being 28%. These experiments make it clear

TABLE 5
RATIO OF CELL INCREASE AND PRODUCTS INCREASE

Data from Table	Organism	Percentage of Cells	Increase of Products
	A. CELL INCREASE EFFECTED BY CROWDING WITH CENTRIFUGED CELLS		
I	baker's yeast	25,000	0
II	Streptococcus A	10,000	11
II	Streptococcus B	30,500	28
	B. CELL INCREASE EFFECTED BY VITAMIN		
III	Lactobacillus casei	90	40
	" "	400	160
	" "	720	240
	" "	1,060	380

that the greater acidity produced by cells fed with vitamin cannot be accounted for merely by the presence of more cells. *Slight* increases in acidity may be explained by the buffering effect of more bacterial protoplasm. The only effect of more enzyme (or more cells) upon any enzymatic reaction or fermentation can be a greater *speed* of reaction. The *endpoint*, however, remains unaffected.

Table 4 has shown that the presence of a few micrograms of riboflavin increases the final amount of acid produced. Since the change cannot be due to a difference in the *number* of cells, it can be due only to a change in the *composition* of the cells, to a change in their acid-tolerance. This viewpoint that the vitamin can affect the tolerance of cells or enzymes to their own products, reverses the common interpretation not only of the vitamin assay, but of our general explanation of vitamin action. Contrary to common conception, the larger amount of acid produced in the presence of vitamin is *not* due to the larger bacterial population. Cause and effect must be reversed. The vitamin-fed cells have become more acid-tolerant, therefore they continue to ferment at an acidity which would block all cells grown without riboflavin. Since these acid-tolerant cells continue to ferment, they continue to furnish available energy to the cells, and consequently, the vitamin-fed cells continue to grow and multiply at a higher acidity, and hence for a longer time than cells raised without riboflavin. In Table 6, for instance, the cells in the culture without riboflavin cease to multiply when the total acidity corresponds to 0.2% lactic acid, certainly before it has reached its maximum of 0.3%. But in the parallel culture, differing only by 2 mg of riboflavin per liter, reproduction is not limited by 0.2% acid, nor is the fermentation. Acid production continues for

TABLE 6
THE EFFECT OF 2 MG RIBOFLAVIN PER LITER ON THE REPRODUCTION AND ACID FORMATION OF *Lactobacillus casei* IN THE DIFCO MEDIUM FOR RIBOFLAVIN ASSAY.

	Control; no vitamin		with 2 ppm riboflavin	
Age of Culture	Total acid as % lactic	Colonies per ml	Total acid as % lactic	Colonies per ml
Start	0.162	7,500	0.162	7,500
13 hrs	0.162	270,000	0.162	260,000
48 hrs	0.180	10,600,000	0.252	116,000,000
59 hrs	0.184	15,000,000	0.405	252,000,000
73-3 days	0.198	18,500,000	0.557	570,000,000
83 hrs	0.207	17,000,000	0.620	650,000,000
96-4 days	0.225	17,000,000	0.683	550,000,000
126-5 days	0.225	16,100,000	0.873	585,000,000
150-6 days	0.207	13,500,000	0.953	495,000,000
174-7 days	0.252	16,600,000	1.070	570,000,000
217-9 days		10,100,000	1.042	605,000,000
270-11 days	0.279		1.17	
385-16 days	0.324		1.26	

a while at a rapid pace, even beyond 0.2%, and multiplication of cells also goes on even at 0.5% acid until finally the vitamin culture has about 30 times as many cells as the parallel culture without riboflavin. The larger crop in the medium containing riboflavin is not the consequence of the presence of a necessary cell-building material; it is the simple result of a longer growth period because the cells are not so easily inhibited by their own fermentation product.

Experimental Proof of Increased Acid Tolerance by Riboflavin. This explanation of the role of riboflavin would remain purely speculative if it could not be proved experimentally. Fortunately, this is possible. Vitamin-fed cells, if more acid-tolerant, should be able to multiply in a medium too acid for reproduction without vitamin.

To six bottles of standard Difco assay medium, various amounts of lactic acid were added so that the added acid concentration amounted respectively to 0, 0.2, 0.4, 0.6, 0.8, and 1.0%. Each batch was divided, and one-half of each received 2 ppm of riboflavin, the other half a corresponding amount of distilled water. The concentration of all other food substances was exactly alike in these 12 batches. After sterilization, the six batches without riboflavin were inoculated from a culture grown without riboflavin; the flasks with riboflavin were inoculated from a parallel culture with riboflavin. The inoculum in both cases was about 100,000 cells per ml. Figure 1 shows how the larger amounts of lactic acid changed the growing cultures into dying

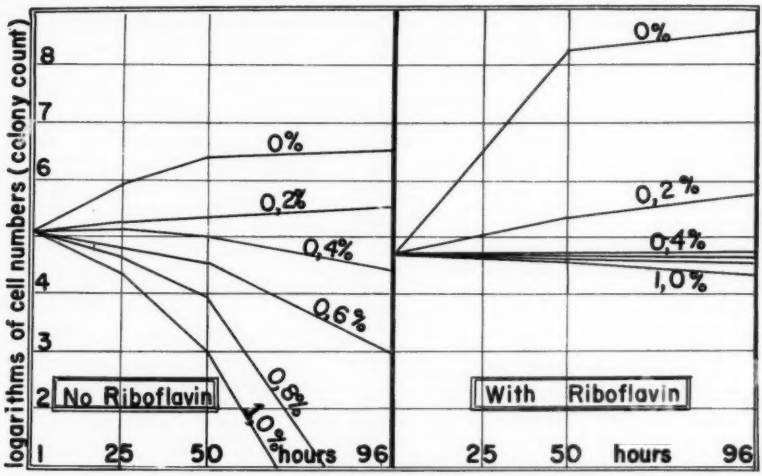

FIGURE 1

Effect of various concentrations of lactic acid upon *Lactobacillus casei* in a good medium without and with riboflavin.

cultures. The presence of riboflavin had a very decided effect on the rates of growth and of death. The better growth with riboflavin in the cultures without lactic acid is nothing new. When 0.2% lactic acid had been added, the cells with vitamin multiplied better than those without it. 0.3% lactic acid seems to be about the limit for reproduction in the absence of vitamin, whereas in its presence, a slight multiplication took place even at 0.4%. This amount is already toxic to the vitamin-free cells, and more acid becomes increasingly toxic to these cells which cannot produce more than 0.3% (see Table 6). Figure 2 presents the same data arranged so that for each amount of added lactic acid, the effect of vitamins is clearly shown. The vitamin-grown cells are quite resistant to acid. Even the addition of 1% caused only a very slow decrease in 96 hours. These cells were capable of producing 1.3% lactic acid with their own enzyme system plus riboflavin. The protective effect of riboflavin against lactic acid is very obvious in these lactic-acid-producing bacteria.

However, the protection seems to cover only the range of action of the lactacidase enzyme as far as it is influenced by its coenzyme. This was tested by suspending 3 days old cells, at the height of their

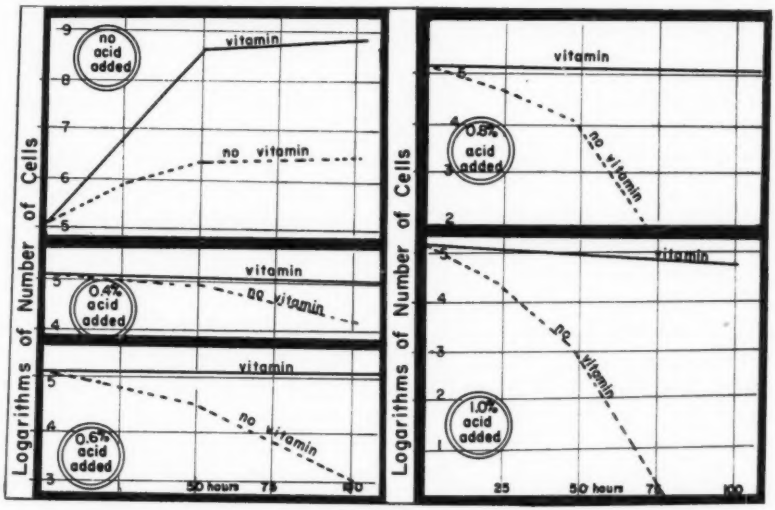

FIGURE 2

Growth and death of *Lactobacillus casei* with and without riboflavin, in a medium with added lactic acid.

development, grown without and with riboflavin, in the assay medium to which 2% or 4% lactic acid had been added. The death rate was measured by plate counts every hour. The cells in 4% acid died more rapidly, but the death rate was independent of whether the cells had been grown without or with vitamin.

We must conclude that though the presence of riboflavin was able to change the tolerance of the apo-enzyme, the vitality or longevity of the cells had not been altered. The ability of the cells to survive an exposure to *high* concentrations of lactic acid, or to recover from injury by more than normal lactic acid, is independent of riboflavin.

This experiment suggests that the vitamin does not influence the entire composition of the protoplasm. More vitamin results in a longer continuation of the energy supply, but this energy is used in the normal way, and the result is not a different type of cells, but merely a larger number of normal cells which differ only by a "protected" apo-enzyme, but not in basic protoplasm. The riboflavin had only affected the acid-forming apo-enzyme, but there is no evidence that any other part of the protoplasm had been changed. This observation may be of importance in explaining the function of vitamins.

The Explanation of the Increased Acid Tolerance is not easy or simple as long as our knowledge of the apo-enzyme of fermentation is so meager. Any fermentation is stopped by the accumulation of its fermentation products whenever an ample supply of fermentable substrate is present. The endpoint of fermentation is brought about by an inhibiting concentration of products. This endpoint is characteristic of the species. A beer yeast may cease to ferment at 6% alcohol, and no addition of sugar will change this endpoint, whereas as a distiller's yeast produces 12% before the fermentation ceases. Cessation means that each individual cell ceases to ferment at this concentration; it means that the fermentation process, the enzyme action producing alcohol and carbon dioxide, is blocked by 6% alcohol in every cell of brewer's yeast, by 12% alcohol in every cell of distiller's yeast. The term "blocking" is used here because it is non-committal. The inactivation of the enzyme is not irreversible. Removal of alcohol (e.g., by vacuum distillation) results in continued fermentation.

The same is true for the lactic fermentation. Different species of streptococci or lactobacilli are blocked at quite different amounts of acid. The inhibiting products are the H ions which can be removed easily by neutralization. By this simple expedient, a culture of *Strept. lactis* which was blocked in milk by 0.7% lactic acid, could be induced to produce 3.2% total lactic acid, after 8 successive neutralizations (Rahn 1932, p. 143). This is the commercial way of obtaining lactic acid.

If a lactobacillus culture without riboflavin is blocked by 0.3% of lactic acid while a parallel culture in the same medium plus riboflavin is capable of producing 1.3% the riboflavin must have changed the sensitivity of the apo enzyme to lactic acid, i.e., to the H ions of lactic acid. We know from the study by Cohen and Rahn (1951) that this increased tolerance to acid is not a simple chemical reaction between vitamin and cell, but is brought about by the growth mechanism of the living cell. Vitamin-deficient cells which are too old to reproduce, are not protected by addition of riboflavin. The composition of the bacterial cells is changed by the incorporation of the vitamin into the apo enzyme. The bacteria remove the riboflavin from the medium, and most of the vitamin (85%) can be recovered from the cells by acid hydrolysis whereas the cell-free, spent medium contains no vitamin (Cohen and Rahn).

We seem to be forced to the conclusion that the enzyme system of

the lactobacillus has become acid-tolerant by incorporation of riboflavin into the apo-enzyme which is most immediately concerned. A direct neutralization of the acid by riboflavin is out of the question considering the minute quantities of vitamins involved, and also, because this vitamin protects only growing cells, but not resting cells. The alternative that not the apo-enzyme as such, but the permeability of the plasma membrane may have been changed by the incorporation of riboflavin into the membrane structure does not seem probable either. A change of the rate of diffusion or of the selectivity of the membrane would affect the *rate* of reaction, but is not likely to change the *final* result which depends always upon the blocking concentration.

The problem resolves itself to the question: How can the incorporation of a substance like riboflavin into a native protein make it more acid-resistant, or more resistant to denaturing agents generally—(The fact that thiamine permits *Lactobacillus fermentum* to make more acid and *Saccharomyces cerevisiae* No. 7753 to make more alcohol, suggests that we are dealing with a general protection against the denaturation of the apo-enzyme).

Any further attempt of explaining the development of acid tolerance can be done only by leaving the solid ground of experimental facts, and venturing onto the deceiving ground of theoretical speculation. We may assume that a reversible denaturation of the apo-enzyme is caused by the disruption of peptide chains, according to Wu (quoted by Haurowitz 1950 p. 127). If we accept the explanation by Wu that denaturation consists of breakage of peptide linkages and eventual recombination in a different way, we can imagine that the vitamin either prevents breakage, or that it effects new and different peptide linkages which make the apo-enzyme more compact and thereby less vulnerable to hydrogen ions, without however interfering with the enzymatic function; the vitamin merely interferes with the easy reaction between the protein, i.e., the apo-enzyme, and the denaturing agent, while the enzyme part remains unaffected.

Growth Rates and Fermentation Rates. The circumstance that cells nourished with riboflavin can tolerate more acid, must have some effect also upon the growth rate. The lactic fermentation, as the only energy source for the synthetic mechanism of the cell, will be less inhibited in the presence of riboflavin, and a more rapid rate of growth must be expected. This is actually the case, as may be seen from

Tables 6, 7, and 8. It is quite significant that the rate of reproduction for the first 13 hours in Table 6 is practically the same with or without riboflavin. In this early stage of multiplication, so little lactic acid is produced that the protection by the vitamin is not needed. We may say that the protection is available, but no use is made of it because only traces of acid are produced. The two cultures behaved therefore identically until the acid accumulated to such an extent that it retarded the culture without vitamin more than the one with it. Identical growth rates of young cultures, independent of the presence of vitamins for a short period, have been repeatedly observed. Only at the later stages of development where hydrogen ions begin to interfere, do the protected, vitamin-fed bacteria multiply more rapidly.

The rate of reproduction and the rate of fermentation was calculated for several similar experiments. The reproduction rate is most conveniently expressed by the generation time, i.e., by the number of hours required for the average cell to double. In the tables, it is called "generation time" or "fission time." The rate of fermentation is measured by the amount of lactic acid produced per cell per hour (Formula for growing cells see Rahn 1932 p. 401). It cannot be computed until the cells have produced measurable amounts of acid. Therefore, no data can be given for very young cultures.

The result for the later stages of development, as long as the cells are multiplying, is given in Table 7 for several experiments. Some (I, II, and X_a) were made with the assay medium as purchased, others (X_s) with the centrifugate from a culture in the assay medium where the bacteria had removed all last traces of vitamin still present in that medium as purchased, and where the bacteria had then been removed by centrifugation; the supernatant liquid was neutralized before sterilization and re-inoculation.

The table shows a very surprising result, namely that bacteria without riboflavin produce more acid per cell per hour than those grown with riboflavin. The differences are quite large, and since the same result was obtained with three experiments, it must be accepted as a fact. The same table gives the doubling time for these cultures, for the identical time periods. As we know how many hours the average cell needs for doubling, and as we know the average amount of acid produced per cell per hour, the product of these two values indicates how much lactic acid a cell must produce to obtain enough energy for doubling. One gram of glucose yields 1 gram of lactic acid;

TABLE 7

GLUCOSE UTILIZATION WITHOUT AND WITH RIBOFLAVIN COMPUTED FOR 0-48 HOURS

Experiment	without vitamin	with vitamin
	GENERATION TIMES	
I	4.6 hours	3.5 hours
II	7.8 "	4.0 "
Xa	6.1 "	3.95 "
Xs	7.65 "	5.15 "
	FERMENTING CAPACITIES (Glucose used per Cell per Hour)	
I	25.6×10^{-10}mg	15.3×10^{-10}mg
II	30.1×10^{-10}mg	23.4×10^{-10}mg
Xa	77.6×10^{-10}mg	17.7×10^{-10}mg
Xs	94.0×10^{-10}mg	8.05×10^{-10}mg
	GLUCOSE USED PER CELL DOUBLING	
	115×10^{-10}mg	52×10^{-10}mg
	235×10^{-10}mg	94×10^{-10}mg
	473×10^{-10}mg	70×10^{-10}mg
	717×10^{-10}mg	42×10^{-10}mg

Xa = Difco Assay Medium for Riboflavin.
Xs = Same after removal of the last traces of riboflavin by bacterial growth.

TABLE 8

REPRODUCTION AND ACID FORMATION BY *Lactobacillus casei* IN A MEDIUM AS FREE FROM RIBOFLAVIN AS POSSIBLE, GIVING ALSO THE FERMENTING CAPACITY, IN MG OF LACTIC ACID PER CELL PER HOUR, AND THE RATE OF REPRODUCTION AS THE TIME REQUIRED FOR THE AVERAGE DOUBLING OF A CELL.

	No Riboflavin				2 ppm Riboflavin			
Age in hours	Colony Count per mm³	% acid lactic produced	F.C. in 10^{-10}mg	Fission Time, in hours	Colony Count per mm³	% acid lactic produced	F.C. in 10^{-10}mg	Fission Time, in hours
0	36	0	——	∞	36	0	——	∞
7	21	0	——	13.0	35	0	——	14.3
20.5	44	0	——	3.6	68	0.009	——	1.85
27	154	0	——	4.6	780	0.018	1.45	2.51
44	1,920	0.009	11.17	11.6	74,000	0.063	2.35	4.42
51.5	3,000	0.010	3.0	∞	240,000	0.122	2.98	12.6
68	1,200	0.018	6.3	"	595,000	0.324	2.95	∞
78.5	3,000	0.025	2.3	"	700,000	0.428	0.92	"
95.5	2,100	0.032	2.0	"	——	0.545	0.93	"
116	1,300	0.040	0.81	"	780,000	0.690	0.66	"
147	1,530	0.045	0.93	"	1,030,000	0.873	0.47	"
171	1,540	0.049	0.93	"	1,210,000	0.999	0.45	"
195.5	1,270	0.053	——	"	780,000	1.107	——	"
212	1,410	0.054	——	"	740,000	1.120	——	"
240	1,800	0.057	——	"	952,000	1.188	——	"
291		0.068	——	"	——	1.283	——	"
316		0.081	——	"	——	1.447	——	"
338		0.086			——	1.380		

TABLE 9
ATTEMPT OF PROVING RIBOFLAVIN ACCUMULATION BY REPEATED GROWTH OF THE LACTOBACILLUS IN THE SAME CULTURE

	A		B		C	
	Cells per ml	% acid	Cells per ml	% acid	Cells per ml	% acid
First Crop	3,400,000	0.117	——	——	——	——
Second Crop	3,045,000	0.144	3,250,000	0.108	——	——
Third Crop	not counted	0.144	3,285,000	0.126	2,335,000	0.135
	after neutralization of acid, sterilization, and re-inoculation					
Start	16,000	0.072	16,000	0.086	16,000	0.086
9 days	1,170,000	0.108	1,440,000	0.117	not counted	0.117

therefore, this product gives also the amount of glucose used in the doubling of one cell. Since $C_6H_{12}O_6 = 2\ C_3H_6O_3 + 28$ calories, the energy requirement for reproduction can be easily calculated. This lactobacillus makes no other product besides lactic acid, nor does it oxidize glucose or its own fermentation products; the above equation describes the fermentation completely. The simplicity of the metabolism, the ease of measuring it by a titration, and the fact that this species does not utilize oxygen, makes it a very useful organism for biochemical experiments because its energy supply can be easily measured. The same is true for most species of Streptococcus.

The last figure of Table 7 brings out an interesting and probably not yet recorded feature of the riboflavin: it saves energy for growth. The cells grown without vitamin need 2 to 8 times as much glucose (or energy) to accomplish a cell doubling. The riboflavin not only protects the lactobacillus against lactic acid; it also functions as a real growth factor, making cell reproduction more rapid and less energy-consuming. The authors cannot explain the energy-saving effect of riboflavin.

Inability of Vitamin Production by the Lactobacillus. The surprising result that a cell without riboflavin ferments more rapidly than one with vitamin, is not easily explained. According to standard conceptions of vitamins as co-enzymes, the cells grown without co-enzymes should not be able to bring about a fermentation at all, unless they can synthesize the riboflavin themselves. This ability is not believed to be possible for the *Lactobacillus casei,* but the experimental facts of Table 7 made it necessary to find out whether, with all other food available, the lactobacillus could synthesize its own vitamin.

The amount of riboflavin synthesized by the cells can be only very small, since the bacteria react promptly upon the addition of a very

small dose. It was tried if, by repeated cultivation of this lactobacillus in a vitamin-free medium, successive generations of bacteria might pile up enough riboflavin to become measurable. The experiment was quite simple. Table 6 has shown that this bacterium multiplies in Difco Assay Medium to about 18 million cells. If these were killed by heat, and the same medium re-inoculated from a vitamin-free culture, the liquid would finally contain the riboflavin synthesized by two times 18 million cells. This could be repeated. Each generation would add more riboflavin, the growth should become better, and finally, it may become necessary to neutralize the acid. The acid increase is not more than 0.3% lactic acid in the 3 days necessary to reach the maximal population, according to Table 7, but if vitamin piles up, more acid will be produced.

This plan was followed, the culture being heat-killed after three days, and re-inoculated without adding riboflavin in the inoculum. This was repeated every 3 to 4 days. Each time when the culture was re-inoculated, a parallel culture in regular assay medium was started to see if a difference was noticeable. The result is shown in Table 9. As neither the acidity nor the maximal number of cells increased significantly, there is no cause to assume that the cells grown in the absence of riboflavin produce measurable amounts of this vitamin.

Summary of Experimental Facts. When riboflavin is added to a culture of *Lactobacillus casei* in a medium which contains everything necessary for the development of the bacteria except riboflavin, the following changes were observed as due to the added vitamin (usually 2 mg per liter):

1) a longer continuation of the lactic fermentation;
2) a larger final amount of the fermentation product, i.e., lactic acid;
3) a larger final number of bacteria;
4) a different type of cell, genetically identical, but individually more tolerant to lactic acid.

Calculations from these Experimental Facts showed:

a) bacteria with riboflavin multiply more rapidly, but only in the later stages of the development of the culture, not when the cells are young and when the accumulating fermentation product, acid, is hardly measurable;
b) young bacteria produce as much or more lactic acid per cell

per hour without riboflavin as the bacteria grown with an ample supply of the vitamin;

c) since bacteria with riboflavin multiply more rapidly, but do not ferment more rapidly, they need less glucose (or energy) for the doubling of a cell, only about ½ to ⅛ of the amount required by cells grown without vitamin.

Conclusions. The main effect of riboflavin upon *Lactobacillus casei* which is hardly capable of multiplying without this vitamin, is an increased tolerance of the bacterium against its own metabolic product, lactic acid. This protection enables the cells which are nourished with vitamin, to maintain the fermenting stage much longer, and to liberate available energy when cells without riboflavin are already inhibited by the same amount of acid. The result is a larger number of cells as well as a higher concentration of acid.

The vitamin-fed cells could be shown to be much more acid-tolerant than the controls. This tolerance is not unlimited. It does not extend to the entire protoplasm, but is limited to the apo-enzyme.

In very young cultures, bacteria multiply with equal rapidity whether riboflavin is present or not, because there is no acid present, and therefore no need for the protective action of the vitamin.

With riboflavin, these bacteria not only multiply more rapidly, but they need less energy, i.e., less glucose per cell doubling. Riboflavin saves energy for growth.

III. Thiamine

The preceding experiments could be explained by the assumption that the cells of *Lactobacillus casei* became tolerant to their own metabolic product, namely lactic acid, by growing in a medium containing riboflavin, a vitamin without which this species cannot grow. The assumption of increased tolerance fitted the data so well that it seemed worth while to test if other vitamins worked by the same principle.

Experimental Proof of Increased Alcohol Tolerance of Yeast by Thiamine. As thiamine is needed by certain yeast varieties, it was tried if the tolerance of these yeasts to their main metabolic product, i.e., alcohol, would be increased by growing the cells in the presence of thiamine. The method of testing was similar to the one used for riboflavin. The yeast variety was the so-called Old Process yeast, American Type Culture Collection No. 7753. The medium was a

synthetic one consisting of minerals, ammonium salt, glucose, and riboflavin. The test was carried out by adding to batches of this medium different amounts of pure alcohol, dividing each batch into halves, and adding to the one half, 0.2 ppm of thiamine, and none to the other half. Aside from the thiamine, the two halves were exactly alike. The batches with thiamine were inoculated from a culture grown with thiamine while the remainder was inoculated with approximately the same number of yeast cells grown without the vitamin.

The results seemed contradictory, and the experiment was repeated twice. The results remained the same: better growth with thiamine, protection against *small* alcohol concentrations, but not against more than 10%. Figures 3 and 4 show that with 7.5% alcohol, the yeast cells with thiamine multiplied, whereas those without it died. With 10% alcohol, the cells with thiamine die, but more slowly than those without it. With 15% alcohol, there is little difference caused by the vitamin, and with 20% alcohol, the cells with thiamine die more rapidly. The decrease was so rapid, however, that the difference may not be significant.

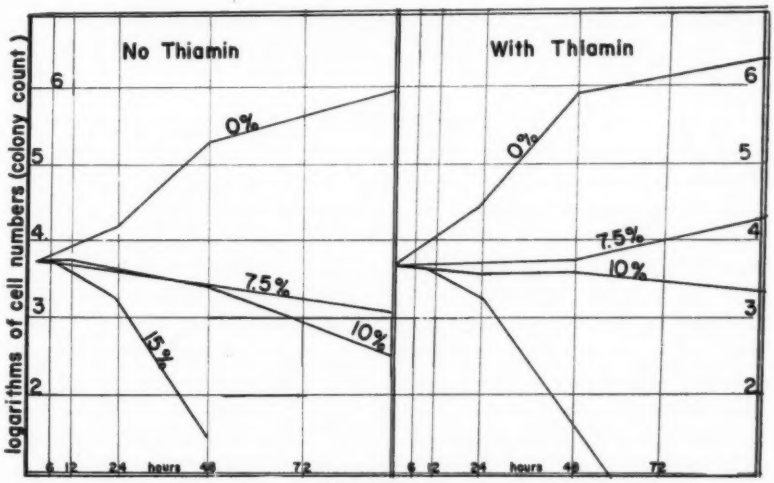

FIGURE 3

Effect of various concentrations of alcohol upon the yeast "Old Process" in a good medium without and with thiamine.

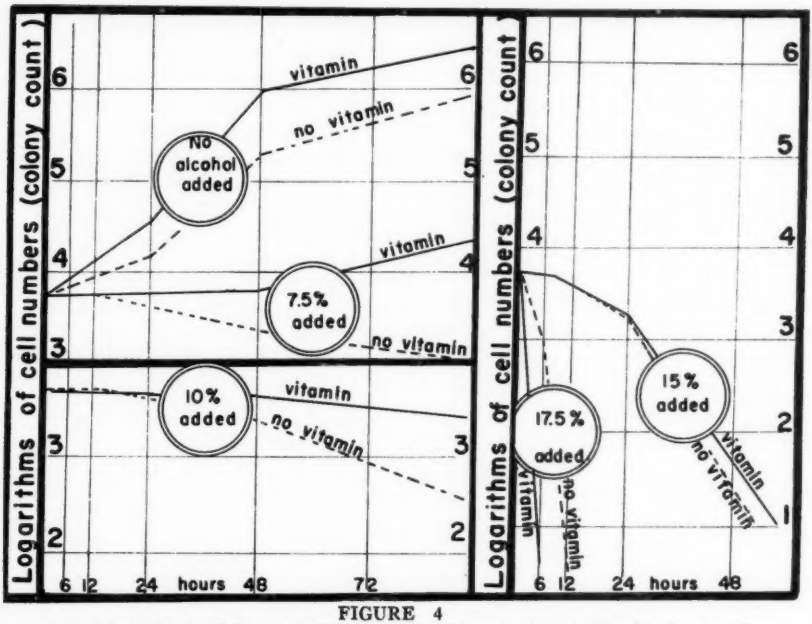

FIGURE 4

Growth and death of the yeast "Old Process" with and without thiamine, in a medium with added alcohol.

On page 275 it was stated that the ability of the lactobacillus to survive *large* doses of acid was independent of the presence of vitamin. The same is observed with yeast and thiamine, and the explanation given there can also be applied here. The protection by vitamins covers only the range of the normal amounts of the fermentation product which for our yeast is about 10% alcohol.* The vitamin makes the yeast enzyme tolerant to the amount of alcohol it can normally produce, but not to higher concentrations. What chemical mechanism comes into action, is not known. Limits of tolerance must be expected, of course. Why these limits coincide with the maximal concentration of fermentation products, is not clear. But it may be, as explained before, that the tolerance extends only to the apo-enzyme, and not to the entire protoplasm. Beyond the endpoint of fermentation, any addition of products must act upon the protoplasm as well as upon the enzyme. This is no more merely a question of energy supply, it is a

*The yeast O.P. produces in this medium 7.5% alcohol by volume if thiamine is present, but only 1.5% without the vitamin.

poisoning of the vital matter of the cell. Even if the apo-enzyme remains protected, it cannot extend this protection to the protoplasm, and the result is death which cannot be retarded by vitamins.

IV. Ascorbic Acid

The conclusion drawn from the preceding experiments was that vitamins are needed by some bacteria or yeasts to protect them (or their apo-enzymes) against the harmful effect of their chief metabolic product. A generalization of the protective function of vitamins is hardly permissible unless it can be shown that it applies to animals as well.

The proof for a protective action of ascorbic acid upon animals can be found in the experiments of Hegarty and Rahn (1938) who studied the lactic acid formation by the muscle of scorbutic guinea pigs. The muscle cells of normal guinea pigs furnished more than 400 mg of lactic acid per 100 g of muscle, whereas the muscle of scorbutic animals produced only half of that amount in the same time. Figure 5 shows that both types of muscle had reached the maximal amount of acid production in twenty minutes. The higher acidity in the normal muscle can be explained simply by a greater acid tolerance of the muscle which had been nourished with the normal vitamin supply which was lacking in the scorbutic muscle. More acid means a larger energy supply which may be used in various ways.

V. Summary

The bacteriological assay of riboflavin is based on the fact that *Lactobacillus casei* does not multiply nor ferment appreciably unless riboflavin is contained in the culture medium. If this vitamin is provided, the organism not only multiplies to large numbers, but produces large amounts of lactic acid.

The usual explanation that riboflavin, as a "growth factor", induces a long period of reproduction, and that the large number of bacteria thus created produces very large amounts of acid, is contradictory to the well-established experience of bacteriology that any fermentation is stopped by a definite concentration of fermentation products, such as acids, alcohol, etc. The limiting concentration is characteristic of the species.

The larger amount of acid produced in the presence of riboflavin is, therefore, not caused by the larger number of cells, but indicates

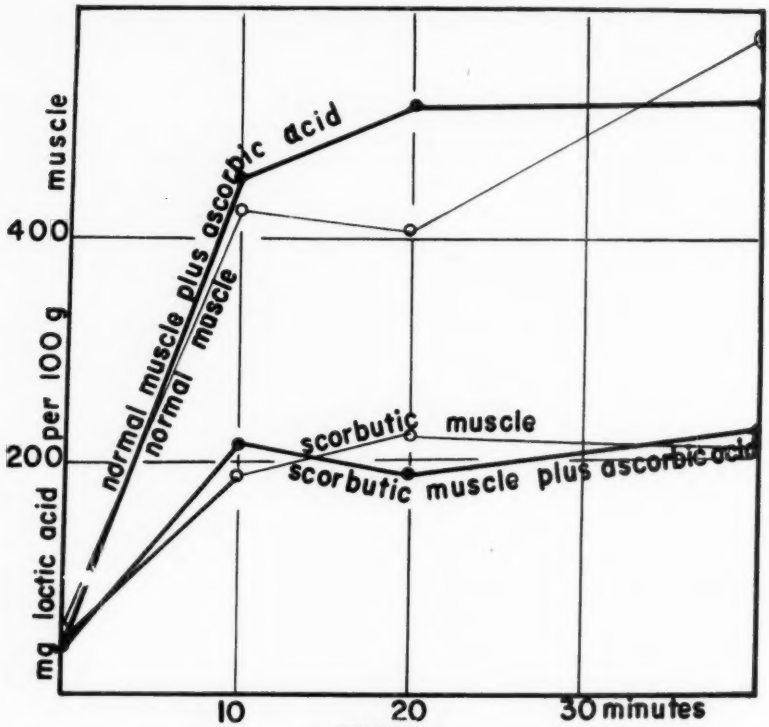

FIGURE 5

Lactic acid formation from glucose by the muscle cells of normal and of scorbutic guinea pigs.

a greater tolerance of the bacteria to the lactic acid. It could be proved experimentally that acid concentrations which prevent reproduction in the vitamin-free medium, are not inhibitive when vitamin is present. Bacteria grown without riboflavin die more readily than those grown with vitamin. The protective effect of vitamins ceases when acid concentrations beyond those produced by normal fermentations are used.

This increased tolerance of bacteria to acid permits them to ferment for a longer time, and thus to furnish energy for a longer time; this results in a longer period of reproduction and, therefore, in a larger final crop of bacteria. This larger crop is not the primary effect of the

presence of riboflavin. The vitamin acts directly on the fermenting enzyme, making it more acid-tolerant, and its longer activity brings about a greater number of bacteria, as a secondary effect.

The same increased tolerance of fermentation products was observed with the "Old Process" yeast which is used for the assay of Vitamin B_1. The yeast cells grown with thiamine were able to multiply in a synthetic medium plus thiamine to which 7.5% alcohol had been added. Without thiamine, the yeast was killed by this amount. These results agree well with Hohl's observations (1938) of the syruped fermentation of wines.

Sixteen years ago, Hegarty and Rahn (1938) studied the acid formation in the muscle of scorbutic guinea pigs. The acid production came to a stop at a low acid concentration, whereas the normal muscle was able to produce about twice as much acid. This different behavior can be explained from the lessons of the preceding experiments as an increased acid-tolerance of the muscle or its enzymes by the presence of ascorbic acid.

From these three experiments the conclusion is drawn that vitamins protect the apo-enzymes in the cells against the harmful effects of their own metabolic products. This conclusion does not contradict the "Co-enzyme Theory"; it merely gives it a more definite meaning.

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*Titles not available at our library.